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MECHANISM OF THE LOCAL VASCULAR ACTIONS OF 1, 1-DIMETHYL-4-PHENYLPIPERAZINIUM (DMPP), A POTENT GANGLIONIC STIMULANT

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Recent reports from this laboratory demonstrated that 1, 1–dimethyl-4-phenylpiperazinium (DMPP), a potent ganglionic stimulant, produced a local vasodilator and a local vasoconstrictor effect when injected intra-arterially into the intact hind limb of the dog (Winbury, Wolf & Tabachnick, 1958; Winbury, 1958). Under normal conditions the dilator action predominated and resisted atropine or acute denervation of the limb; the vasoconstrictor action was observed only after ganglionic block with hexamethonium. Burn & Rand (1958), however, observed only a constrictor response in the isolated perfused hind limb of the dog, and Page & McCubbin (1953) reported that DMPP produced no effect in the same preparation.

Nicotine, another ganglionic stimulant, has a direct vasoconstrictor effect in the isolated rabbit ear and in the hind limb of the dog; these effects were blocked by hexamethonium or by pre-treatment with reserpine (Kottegoda, 1953; Ginzel & Kottegoda, 1953; Burn & Rand, 1958). The response was attributed to the release of norepinephrine from chromaffin tissue in or near the blood vessels. In fact, Burn & Rand (1958) demonstrated that the decline in the vasoconstrictor activity of nicotine following reserpine coincided with a decline in the norepinephrine content of the blood vessels. Hilton (1954), working with the hind limb of the cat, reported that nicotine can elicit both a vasodilator and a vasoconstrictor response in the muscle of the hind limb of the cat via axon reflexes in cholinergic or adrenergic fibres, respectively. Folkow (1955, 1956) questions the conclusions of Hilton (1954) and suggests that if such an axon reflex is present it differs from those previously described.

With this background to nicotine and DMPP we decided to analyse further the mechanisms of the local vascular actions of DMPP by use of various types of blocking agents and by chronic denervation. The experiments to be described suggest that the dilator and constrictor actions of DMPP in the innervated limb are mediated by distinct nerve pathways, and that after chronic total denervation of the limb DMPP exerts a direct relaxant action on the smooth muscle of the vessel wall.

METHODS

These studies were carried out in 56 dogs, measuring the total blood flow in one or both hind limbs. The majority of animals were anaesthetized with sodium thiopentone, 20–30 mg/kg I.V. followed by α-chloralose, 60–80 mg/kg I.V. Some animals were anaesthetized with sodium pentobarbitone, 30 mg/kg I.V. Additional anaesthetic was used as required to maintain a steady level of blood flow.

The blood-flow rate (inflow) was recorded with a rotameter (Shipley & Wilson, 1951) connected between the central end of a cut femoral or carotid artery and the peripheral end of a cut femoral artery. Heparin, 1500 u./kg, was administered 5–10 min before cannulation. Pontamine Fast Pink BL, 10–25 ml. of a 5% solution, was used to enhance the anticoagulant action (Winbury, Michiels, Hambourger, Stockfisch & Cook, 1950). The pressure in the rotameter circuit was determined with a pressure transducer at the inflow tubing close to the inflow cannula and was recorded simultaneously with the blood-flow rate on a direct-writing electronic recorder. In a few experiments the blood flow to the muscle was measured separately (no skin) according to the procedure of Lanier, Green, Hardaway, Johnson & Donald (1953).

All intra-arterial injections were administered into the outflow tubing of the rotameter circuit (Winbury *et al.* 1950) close to the outflow cannula. Compounds were dissolved in NaCl solution 0·9% (w/v) and injection volumes were kept below 0·2 ml. Intravenous injections were given into a cannulated jugular vein.

In several animals one hind limb was totally denervated 7–12 days before the experiment by cutting the sciatic plexus and femoral nerve and stripping the adventitia from the femoral artery. When the flow studies were performed, the femoral artery in the denervated limb was cannulated distal to the operative site.

RESULTS

Intact innervation

Control responses to DMPP and nicotine. Intra-arterial injection of DMPP (100–300 μg total dose) usually resulted in a prompt increase in blood flow, similar to that observed after nicotine (20–200 μg) or acetylcholine (ACh, 0·1–1 μg) (Figs. 1–5). Similar dilator effects were noted in the few experiments on muscle blood flow. In a few animals a vasoconstrictor response to DMPP was observed at the start of the experiment but this changed to a dilator response within a short period of time. Constrictor responses were not observed with nicotine or ACh.

Effect of ganglionic blockade: reversal of DMPP and blockade of nicotine. After hexamethonium 2 mg/kg I.V. the dilator response to nicotine was blocked while the dilator response to DMPP was changed to a *constrictor* response, (Figs. 1, 4). The response to ACh was not affected by hexamethonium.

After prolonged periods of ganglionic blockade a diminished dilator response returned for both DMPP and nicotine. However, since the constrictor action of DMPP was still present, a two stage response resulted with DMPP, i.e. a slight transient dilatation followed by the more prolonged constriction. Additional doses of hexamethonium did not block this reduced dilator action of nicotine or DMPP. In a few experiments atropine 1 mg/kg I.V. eliminated the reduced dilator response to nicotine but had no effect on the DMPP dilatation.

Effect of cholinergic blockade: elimination of ACh dilator response. Atropine 1 mg/kg I.V. completely blocked the dilator effect of ACh (Fig. 1). The effect of atropine on DMPP and nicotine was variable. In the majority of experiments atropine had little effect on the DMPP or nicotine dilator action; in a few cases, however, there was a slight reduction of the dilator action. In no case was the dilator response to nicotine or DMPP eliminated by atropine.

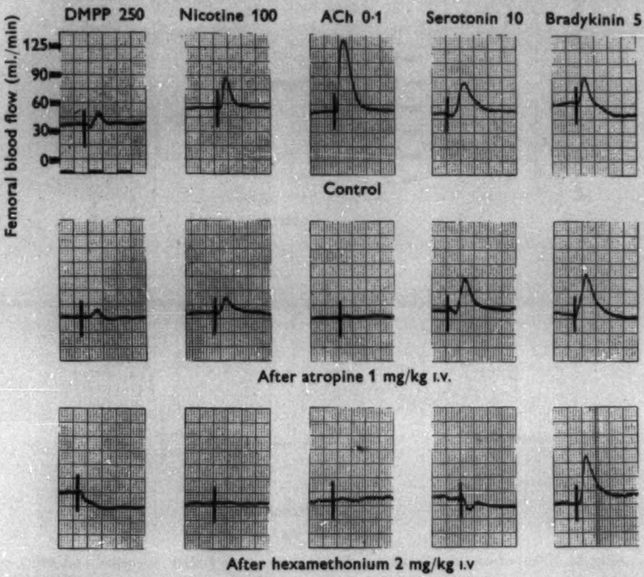


Fig. 1. Effect of atropine and hexamethonium on the vasodilator response to DMPP, nicotine, acetylcholine (ACh), serotonin, and bradykinin; doses in μg. Ordinate, changes in the rate of blood flow (ml./min) in the femoral artery of the dog. The heavy vertical bars indicate the points of injection; time marker 20 sec.

Effect of neostigmine on the dilator response. A dose of neostigmine 10 μg/kg I.V. markedly enhanced the dilator response to ACh but not that to DMPP or nicotine. Higher doses of neostigmine actually decreased the response to DMPP but not that to nicotine.

Effect of cocaine on the response to DMPP and nicotine. The blockade of the dilator response to nicotine and DMPP by hexamethonium suggested that 'ganglionic-like' stimulation (in the periphery) or some related phenomenon may be involved. Cocaine, which blocks nerve transmission (Goodman & Gilman, 1955), was employed further to evaluate the dilator mechanism.

Doses of 5–20 mg were administered by the intra-arterial route in one limb and the flow immediately interrupted for 1–2 min. The other limb served as a control.

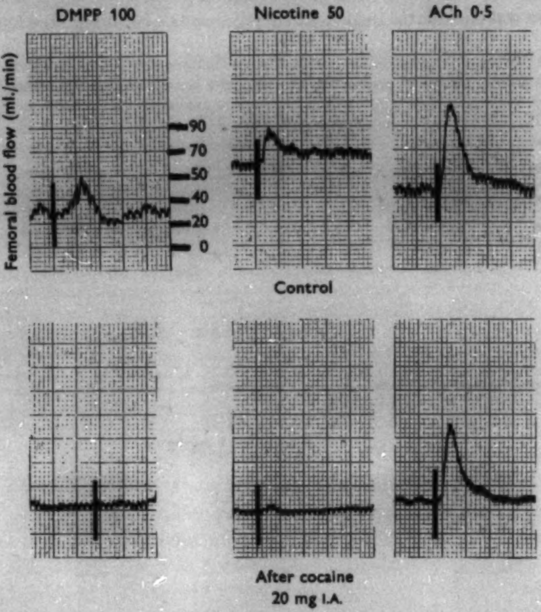


Fig. 2. Effect of cocaine on the vasodilator response to DMPP, nicotine, and ACh. Conventions as for Fig. 1.

The vasodilator action of DMPP was temporarily blocked (15–20 min) by doses of cocaine ranging from 10 to 20 mg (Fig. 2). However, reversal to a constrictor effect was not observed. Smaller doses of cocaine produced only partial blockade. Nicotine was also blocked, but in some cases a small residual response persisted. ACh was not affected by cocaine.

Effect of botulinum toxin on dilator response to DMPP and nicotine. Botulinum toxin prevents transmission in motor nerves as well as autonomic *cholinergic* nerves by blocking the smaller terminal branches close to the myoneural junction (Brooks, 1954, 1956; Wright, 1955; Ambache, 1955). Type A or type C toxin (2×10^6–10^7 mouse lethal doses) was injected into a femoral artery and the flow interrupted for several minutes.

About 2–3 hr after the injection the dilator response to DMPP and nicotine was blocked in both limbs. In approximately half the animals DMPP now produced a constrictor response (Fig. 3) but no constrictor response was observed with nicotine. The dilator response to ACh was not altered or was reduced slightly. Thus botulinum toxin altered the response to DMPP in the same general way as hexamethonium by specifically blocking the dilator action.

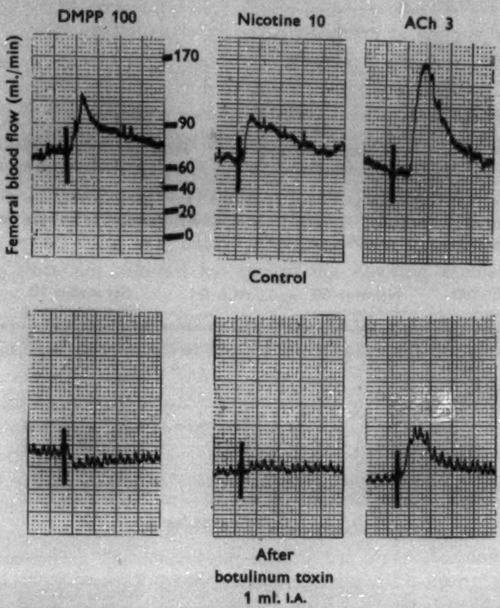
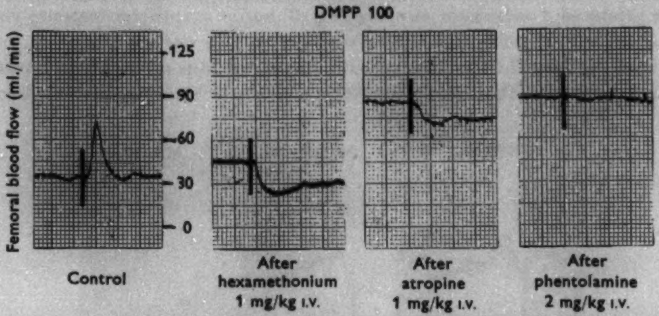


Fig. 3. Effect of botulinum toxin on the vasodilator response to DMPP, nicotine, and ACh. There was an interval of about 2 hr between the control and test records. Conventions as for Fig. 1.

Effect of various agents on the constrictor response to DMPP. Attempts were made to study the mechanism of the constrictor action of DMPP by administration of phentolamine, cocaine, atropine or large doses of hexamethonium. Before administration of these agents it was necessary to block the dilator action of DMPP with hexamethonium 2 mg/kg I.V.

Phentolamine 2 mg/kg I.V. eliminated the constrictor response (Fig. 4). This dose of phentolamine produced a reversal of the constrictor action of epinephrine, and markedly reduced the constrictor action of norepinephrine. Intra-arterial injection of cocaine also blocked the constrictor action of DMPP but did not affect the response to epinephrine or norepinephrine. On the other



Control

After hexamethonium 1 mg/kg I.V.

After atropine 1 mg/kg I.V.

After phentolamine 2 mg/kg I.V.

Fig. 4. Effect of atropine and phentolamine on the vasoconstrictor response to DMPP. The constrictor response to DMPP (panel 2) was observed after blockade of the dilator response by hexamethonium. Conventions as for Fig. 1.

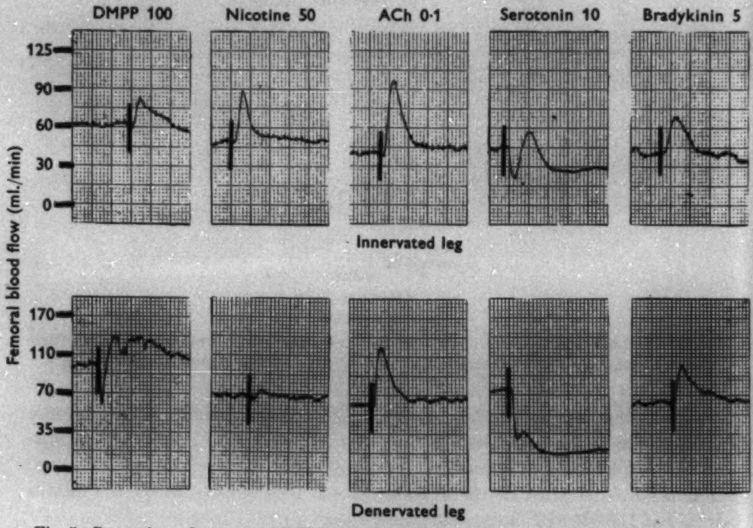


Fig. 5. Comparison of response to DMPP, nicotine, ACh, serotonin, and bradykinin in normal and chronically totally denervated limb; denervation 7 days before experiment. Conventions as for Fig. 1.

hand, the constrictor action of DMPP was not altered by increasing the dosage of hexamethonium to a total of 8 mg/kg or by administration of atropine 1 mg/kg I.V. (Fig. 4).

Effect of reserpine on the response to DMPP and nicotine. Burn & Rand (1958) demonstrated that reserpine pre-treatment depleted the peripheral stores of norepinephrine and prevented the constrictor actions of nicotine and DMPP. In order to ascertain whether or not a local release of catecholamines might be involved in the present results, animals were pre-treated with reserpine 0·5–2 mg/kg intraperitoneal on each of 2 days before the flow study.

Reserpine pre-treatment did not alter the constrictor or the dilator response to DMPP or the dilator response to nicotine. As in the non-reserpinized animal, hexamethonium blocked the DMPP and nicotine dilatation and permitted the DMPP constrictor response to appear; this was blocked by phentolamine. The pressor response to carotid occlusion was absent in the reserpinized animals, suggesting that an adequate dose of reserpine was used.

Effect of other vasoactive agents on blood flow. Several other types of agents were studied for comparative purposes. The following substances consistently produced a dilator response like that of DMPP, nicotine or ACh; isoprenaline 5 μg, histamine 1–5 μg, or bradykinin 5–10 μg. The dilator actions of these substances were not affected by any of the blocking agents previously discussed.

Serotonin 5–10 μg, like DMPP, appeared to have both a dilator action (Fig. 1) and a constrictor. These actions may appear alone or there may be a combined response (Fig. 5). The dilator action of serotonin was blocked by hexamethonium (Fig. 1) and botulinum toxin, but the constrictor response was not altered by any of the blocking agents studied.

Epinephrine (1 μg) and norepinephrine (1 μg) consistently produced a constrictor response, which was reduced or converted to a dilator response by phentolamine.

Chronic total denervation

Control responses. The control responses in the denervated and innervated limbs are shown in Fig. 5. DMPP produced a greater and more prolonged dilator response in the denervated limb than in the innervated limb; in some animals the augmented dilator response was preceded by a transient constrictor action. In contrast, nicotine had a markedly reduced response in the denervated limb, and in several animals, one of which is illustrated in Fig. 7, there was practically no response. The dilator action of serotonin was also blocked by denervation. ACh and bradykinin produced the same effect in both limbs.

Effect of various blocking agents. The dilator effect of DMPP in the denervated limb was *not* blocked by hexamethonium, atropine, phentolamine, botulinum toxin or cocaine (Figs. 6, 7). The results for the first three autonomic blocking agents, given sequentially, are illustrated in Fig. 6. The reduced dilator responses for DMPP, ACh, and bradykinin after hexamethonium are probably associated with the marked reduction in blood pressure. These responses increased as the pressure increased. However, the blockade of the slight dilator effect of nicotine appears to be real, since the response did not return when the perfusion pressure increased. Atropine produced a slight diminution of the DMPP dilator response and completely blocked the ACh effect. Phentolamine produced no further change. Bradykinin and serotonin were not affected by any of these agents.

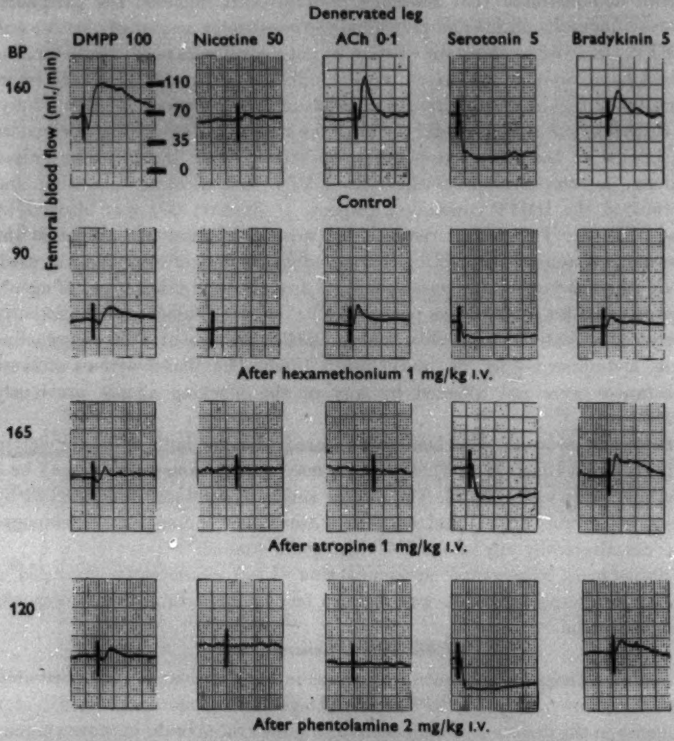


Fig. 6. Effect of hexamethonium, atropine, and phentolamine on the vascular response to DMPP, nicotine, ACh, serotonin, and bradykinin in the chronically (7 days) denervated limb. The blood pressure (mm Hg) during each phase of the experiment is given on the left. Conventions as for Fig. 1.

These blocking agents produced the usual effects in the contralateral innervated limb. Hexamethonium reversed DMPP, and blocked nicotine, vasodilator responses; atropine blocked ACh dilatation. It is obvious the dilator action of DMPP has a different mechanism after chronic denervation. This is not the case for ACh, bradykinin, and the small residual action of nicotine.

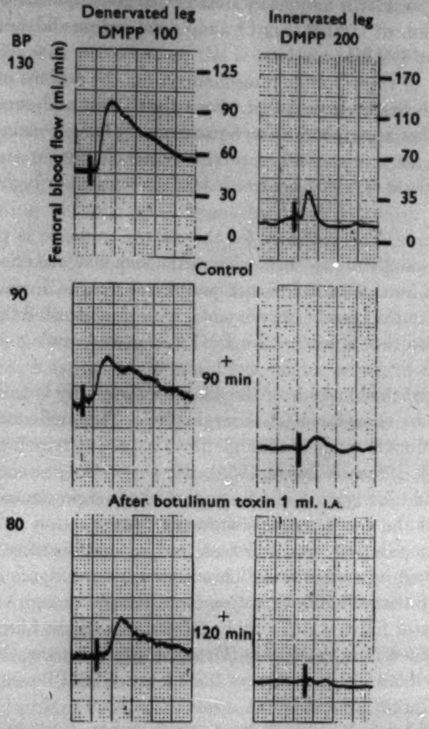


Fig. 7. Effect of botulinum toxin on the vasodilator response to DMPP in the normal and (7 days) denervated limb. Conventions as for Fig. 1. Note change in ordinate scale.

DISCUSSION

These studies showed that DMPP has both a dilator and a constrictor action, on intra-arterial administration, which are mediated by different neural pathways. The dilator action usually predominated under control conditions and the constrictor action was observed only after elimination of the dilator action.

The most significant observations are as follows: (1) The dilator response to DMPP was converted to a constrictor response by hexamethonium or botulinum toxin. (2) The dilator response to DMPP was blocked by cocaine but no constrictor response appeared. (3) The constrictor response to DMPP was blocked by phentolamine or cocaine. (4) The constrictor response was not blocked by hexamethonium or reserpine. (5) Atropine did not markedly alter the constrictor or dilator actions of DMPP. (6) Neostigmine did not enhance the dilator action of DMPP. (7) Chronic denervation did not eliminate the dilator action of DMPP.

On the basis of these results a mechanism for the actions of DMPP in the innervated limb has been proposed. Both the dilator and constrictor actions appear to involve a peripheral nerve pathway, because cocaine blocked both actions. Since acute denervation produced no change in the responses to DMPP (Winbury *et al.* 1958) central mechanisms (cord or higher reflexes) can be excluded.

Dilator response. The pathway for the dilator response is presumed to be activated in a 'ganglion-like' fashion since the response was effectively blocked by hexamethonium. Whether or not peripheral ganglia in fact exist cannot be answered with the present experiments, but it is suggested that there is some hypothetical site that is excited by DMPP in a way analogous to ganglionic stimulation.

On the basis of the blockade of the dilator response by botulinum toxin this pathway may be considered 'cholinergic-like'. The evidence for a selective action of botulinum toxin on cholinergic fibres is quite convincing (Wright, 1955; Ambache, 1955; Hilton & Lewis, 1955). However, the present studies do not provide evidence for release of the typical cholinergic neurohumoral transmitter (ACh) at the nerve endings, since the dilator action of DMPP was not blocked by atropine nor was it potentiated by neostigmine. Acetylcholine itself was blocked by atropine and potentiated by neostigmine.

It is unlikely that the dilator action is mediated through the sympathetic cholinergic dilator fibres which supply the muscles of the hind limb, because this pathway is sensitive to atropine (Uvnäs, 1954; Youmans, Green & Denison, 1955; Folkow, 1956). In many ways the dilator of DMPP resembles the vasodilatation produced, by antidromic stimulation, in the skin (not the muscle) of the hind limb of the cat (Folkow, 1955, 1956) and in the isolated sympathectomized rabbit ear (Holton & Perry, 1951; Holton, 1953). The vasodilatation in both these preparations was not blocked by atropine, and in the studies on the rabbit ear the vasodilatation was reduced by neostigmine and other cholinesterase inhibitors.

Constrictor response. The constrictor mechanism is postulated to be mediated via an adrenergic pathway which is not excited by DMPP in a 'ganglion-like' fashion. In contrast to the dilator action, the constriction was not affected by ganglion blockade.

The site of blockade of the constrictor response of DMPP by cocaine is central to the neuro-effector junction, since epinephrine produced the normal constrictor response. After adrenergic blockade with phentolamine the constrictor action of DMPP was blocked and that of epinephrine was reversed. Doses of reserpine which have been reported (Burn & Rand, 1958) to deplete the norepinephrine stored in chromaffin tissue and arterial walls and to prevent the constrictor response to nicotine and DMPP in the isolated hind limb of the dog, were without effect on the constrictor response to DMPP in our studies.

Chronic denervation. DMPP produced an enhanced dilator response in the chronically denervated limb compared with the contralateral innervated limb. Of greatest interest is the fact that the response to DMPP was not blocked by hexamethonium, cocaine, botulinum toxin, atropine or phentolamine, suggesting an entirely different mode of action in the denervated limb.

It seems most likely that the dilator action of DMPP in the denervated limb results from a direct action on the smooth muscle of the vessel walls similar to that of bradykinin or nitroglycerine. It has been well established that denervation may increase the sensitivity of various organs to sympathomimetic amines; a sensitization to a direct dilator action of DMPP may also result. Presumably this mechanism was of little significance in the innervated limb and the dilatation resulted from the atropine-insensitive neural mechanism.

There is some evidence for a direct dilator action in the innervated limb. For example, in some animals under prolonged ganglionic blockade a slight transient dilator response reappeared, which could not be blocked by larger doses of hexamethonium or by atropine. Further, in studies in which phentolamine blocked the constrictor action of DMPP (after ganglionic blockade), a slight dilator response occasionally reappeared and this could not be blocked by more hexamethonium or atropine.

There are many similarities in the dilator actions of DMPP, serotonin, and nicotine; all three substances were blocked only by hexamethonium and botulinum toxin. However, after chronic denervation differences appeared; the dilator response to serotonin and nicotine was absent but not that to DMPP. There are also differences in the constrictor mechanism for DMPP and serotonin; the action of DMPP, but not that of serotonin, was blocked by phentolamine or cocaine. The reduction in the dilator response to nicotine after chronic denervation is in agreement with the results which Hilton (1954) obtained on the muscles of the hind limb of the cat.

Other compounds classified as ganglionic stimulants, such as tetramethylammonium and the imidazole-acetylcholines, resemble acetylcholine in that the dilator response is blocked by atropine but not by hexamethonium (Winbury, 1957, 1958; Winbury *et al.* 1958).

The following conclusions have thus been drawn about the dilator and constrictor actions of DMPP:

(1) Distinct nerve pathways are involved in the dilator and constrictor actions of DMPP in the innervated limb.

(2) The dilator action indicates the existence of an atropine-insensitive pathway (cholinergic ?) that can be blocked by hexamethonium and botulinum toxin but is not potentiated by neostigmine.

(3) The constrictor action indicates the existence of a pathway (adrenergic) that can be blocked by phentolamine but not by hexamethonium.

(4) Normally the dilator action of DMPP predominated.

(5) There may be a slight direct action of DMPP on the vessel wall, which is masked by 2 and 3 in the innervated limb.

(6) After chronic denervation the limb becomes sensitized to the direct dilator action of DMPP.

SUMMARY

1. The mechanisms of the local vascular actions of 1, 1-dimethyl-4-phenylpiperazinium (DMPP) in the hind limb of the dog were investigated by means of various blocking agents and chronic denervation, and compared with those of nicotine and acetylcholine.

2. DMPP usually produced a local dilator response on intra-arterial injection but vasoconstriction was occasionally observed at the start of the experiment and later changed to vasodilatation.

3. The dilator action of DMPP was converted to a constrictor action by hexamethonium and botulinum toxin. Cocaine blocked the dilator response but no constrictor response appeared; atropine and pre-treatment with reserpine had no significant effect on the dilator response. Neostigmine did not potentiate the dilator response.

4. The constrictor response to DMPP which appears after hexamethonium was blocked by phentolamine or cocaine but not by atropine, pre-treatment with reserpine or large doses of hexamethonium.

5. After chronic total denervation DMPP produced an enhanced dilator response which was not blocked by hexamethonium, botulinum toxin, cocaine, atropine or phentolamine.

6. Nicotine produced a local dilator response which was blocked by hexamethonium, botulinum toxin or cocaine but not by atropine. No constrictor response was observed. After chronic denervation nicotine produced no local vascular effect.

7. Acetylcholine produced a local dilator response which was blocked by atropine but not by hexamethonium, botulinum toxin, cocaine or chronic denervation.

8. It was concluded that the vascular actions of DMPP in the innervated limb involved distinct nerve pathways within the limb. The dilator pathway

is sensitive to botulinum toxin but the neurohumoral transmitter does not appear to be cholinergic. The constrictor pathway appears to be adrenergic in nature. The dilator response after chronic denervation would appear to result from a direct action on the vessel.

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OXIDATION-REDUCTION CHANGES OF CYTOCHROMES FOLLOWING STIMULATION OF AMPHIBIAN CARDIAC MUSCLE

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Previous work carried out in this laboratory on aerobic skeletal muscle (Connelly & Chance, 1954; Weber, 1957; Jöbsis & Chance, 1957) demonstrated changes in the components of the electron transport chain in response to contractions. This paper reports similar studies with the cardiac muscle of the toad and the frog. The only previous observations on the heart are those of Arvanitaki & Chalazonitis (1947). The technique used (filters and a single-beam instrument) does not allow the elimination of the non-specific changes, which makes their conclusions not completely definitive.

The cardiac muscle has a high working capacity, that is it can immediately increase its working level and remain there without showing fatigue. Hence it is an ideal material for studying the control mechanisms operating on metabolism. It is known, for instance, that the time course of the oxygen consumption of the heart, studied by polarographic (Ramírez, 1953) or spectrophotometric methods (D. W. Lübbers & J. Ramírez, unpublished) follows the cardiac work without delay when it is modified by changes in the load or in the frequency of contractions.

In the present study the changes of the steady-state oxidation–reduction levels of the cytochromes, pyridine nucleotides, and flavo-protein in the cardiac muscle were followed during and after isometric contractions by means of spectrophotometric techniques as a further step in the understanding of the control of the cardiac respiratory mechanism. The hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and inorganic phosphate (P) as a consequence of muscular contraction has been postulated (Szent-Györgyi, 1951), but the direct chemical techniques applied to muscle have so far yielded only negative results (Fleckenstein & Janke, 1953; Mommaerts, 1955). On the other hand, the use of spectrophotometric techniques for the indirect analysis of the ADP inside the muscle (Chance & Connelly, 1957) has the great advantage of not destroying the muscle structure. One may use the respiratory chain as a qualitative rapid indicator of the intracellular modifications in the ADP concentration, since the ADP is highly specific in its action on the respiratory chain (Chance & Williams, 1956*a*).

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For comparison, the sarcosomes, which contain the respiratory enzymes under study, were isolated from the cardiac muscle and the steady-state changes in the respiratory chain components which occur in response to the addition of ADP were recorded (Chance & Baltscheffsky, 1958). The addition of ADP in these experiments is assumed to simulate a muscular contraction. A preliminary account of part of this work has been given previously (Ramírez, 1958).

METHODS

The hearts of the toad (*Bufo marinus*) and the frog (*Rana pipiens*) were used. The animal was prepared by injecting sodium heparin (50 mg/kg) into the dorsal lymphatic sinus. After 15 min the animal was killed by destroying the brain and medulla with a needle, and then perfused with 500 ml. of frog's Ringer (mM: NaCl, 111·4, KCl 2·7, $CaCl_2$ 1·8, Na phosphate buffer 2, pH = 7·1) in order to wash out the blood, which might interfere later in the spectrophotometric measurements. The heart was excised and a piece of ventricular wall was mounted, compressing it gently between two perforated lucite plates (Fig. 1), leaving uncovered a 'disk' of stretched muscle of 5·5 mm diameter. The thickness of the excised tissue, between 0·5 and 1·5 mm, is not completely uniform owing to the spongy internal structure of the ventricle. The preparation was put in an ordinary 1·0 cm cuvette in the spectrophotometer and a continuous jet of oxygenated Ringer's solution was directed on each muscular surface to avoid anoxia during the measurements. Such a preparation does not show spontaneous activity in most of the experiments. To elicit the activity the muscle strip was stimulated electrically by means of condenser discharges. The perfusion liquid and the preparation were maintained at constant temperature throughout the experiment. The difference spectra of the heart strips were obtained in the split-beam spectrophotometer described by Yang & Legallais (1954).

In order to study the kinetics of the oxidation–reduction changes of the different components in response to changes in contractile activity, the double beam spectrophotometer described by Chance (1951) was used. The light was detected by an 'end-on type' photomultiplier (Dumont 6292) located at 3·3 cm from the muscle surface. At this distance the large sensitive surface (15 cm^2) of this tube picks up most of the light scattered by the preparation. The difference in optical absorption at two wave-lengths (less than 20 mμ apart) was recorded as a function of time with a Hathaway high-frequency mirror-galvanometer oscillograph (model S 14-A). An Esterline-Angus recorder was used for monitoring purposes. By this differential technique we cancel out most of the non-specific optical changes due to reflexion, polarization, diffraction, and scattering, which occur during the contractions (Hill, 1953), except in the Soret region of the spectrum where these changes are wave-length-dependent. However, if one waits until the contractions cease, the non-specific changes disappear, and then it is possible to record the modifications of the pigments as in the case of cytochrome a_3 (*vide infra*). With these procedures the spectral changes corresponding to the oxidation–reduction of the respiratory chain components can be studied. If a plate of ground glass is put in front of the preparation in order to diffuse the incident light, one can further eliminate the non-specific optical changes originating during the contractions. The non-specific changes recorded at a single wave-length are five to twenty times bigger than the modifications of the spectra of the respiratory pigments. At the reference wave-length, where practically only the non-specific changes are present, the decrease of the optical transmittance due to each contraction was used as a convenient measure of the contraction. These changes are bigger than in skeletal muscle, because in the heart the contractions are taking place in a muscle with highly irregular fibre disposition and heterogeneous transmittance. The sarcosomes were prepared from toad's heart according to the method described by Cleland & Slater (1953) and suspended in the magnesium-free medium described by Packer (1957). The oxygen consumption was measured with the platinum oxygen electrode (Chance & Williams, 1955*a*).

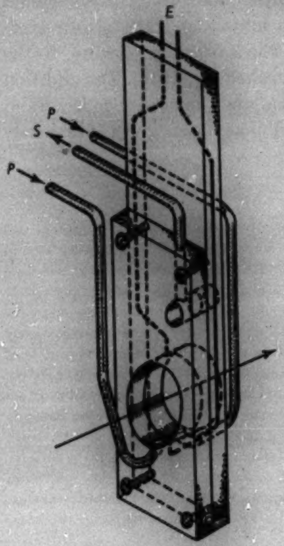


Fig. 1

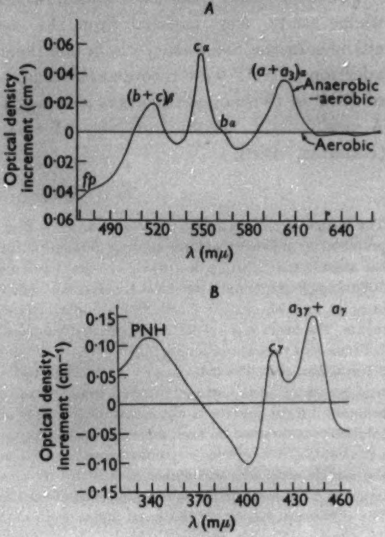


Fig. 2

Fig. 1. Chamber for cardiac muscle. The muscle (not shown) is put between the two perforated lucite plates. These plates are held together with four screws. *E* indicates the stimulating electrodes, *P* the tubes carrying oxygenated Ringer's solution to each muscle surface, and *S* the suction output for Ringer's solution. The whole chamber is put in a 1·0 cm cuvette. The large arrow shows the direction of the spectrophotometer light beam.

Fig. 2. Difference spectrum of oxygenated minus anoxic toad's ventricle. The base line represents the 2 strips oxygenated. *A*. α and β bands of the cytochromes and flavoproteins (fp). *B*. Soret (γ) band of the cytochromes and reduced pyridine nucleotides (PNH). For explanation see text.

RESULTS

Resting state absorption spectra

The difference spectrum of the toad heart strips recorded with the split-beam spectrophotometer is shown in Fig. 2 as the difference between the aerobic and the anaerobic states. Two strips prepared so as to be as similar as possible were gassed with oxygen or nitrogen and the difference spectrum was recorded. The base line corresponds to both muscles being in the aerobic steady state. In the anaerobic-minus-aerobic spectrum, the peaks and troughs corresponding to the difference between the reduced and oxidized pigments of the respiratory chain are apparent. The peak at 605 mμ $(a+a_3)$ α corresponds mainly to the α peak of cytochrome *a* with a small contribution of cytochrome a_3 (Chance, 1957). The shoulder around 564 mμ (*b*α) corresponds to the α band of cytochrome *b*. At 550 mμ (*c*α), the α band of cytochromes $c+c_1$, is seen. The β bands of cytochromes $c+b$ appear at 518 mμ. A trough at 465 mμ corresponds to flavoprotein (*fp*) and is followed by the γ band of cytochrome a_3 at 445 mμ. The γ band of cytochrome *b* is masked by the γ bands of the other cytochromes. The γ band of cytochrome *c* is apparent at 419 mμ, and the peak of the reduced pyridine nucleotides (PNH) is found at 340 mμ. It is evident that the toad's heart does not contain a measurable amount of myoglobin.

Figure 3*A* shows the difference spectra of the frog's heart recorded similarly. In this case there is no clear indication of the reduced cytochrome peaks, as in the toad. Instead, peaks are evident at 600, 557 and 513 mμ, and two troughs at 557 and 537 mμ. This is rather characteristic of the haemoglobin or myoglobin differential spectra but the position and size of the peaks is different. This spectrum can be resolved into two main components, the spectrum of the respiratory-chain components and the spectrum of an intracellular myoglobin-like pigment. The spectrum of the respiratory chain was evidenced by reducing the respiratory pigments of one muscle by adding 1–2 10^{-3} M-NaCN and bubbling oxygen to both strips. Under these circumstances, the intracellular myoglobin-like pigment is oxygenated in both muscles and in the differential trace its spectrum is cancelled out. Only the characteristic peaks of the respiratory chain, similar to those of the toad's heart spectrum, then appear (Fig. 3*B*). The intracellular pigment was recorded without the interference of the peaks of the respiratory pigments, by adding NaCN to both muscles and passing nitrogen to one strip and oxygen to the other. By this procedure the respiratory chain is reduced in both muscles and its spectrum disappears in the differential trace, leaving only the spectrum corresponding to the reduced-minus-oxygenated forms of the intracellular pigment (Fig. 3*C*). A similar trace is obtained also by subtracting the spectrum 3*B* from the spectrum 3*A*. This trace is very similar to the spectrum of the oxygenated-minus-reduced frog's haemoglobin in the erythrocytes. The positions of the peaks and troughs are practically identical (Table 1). This intracellular 'myoglobin-like' pigment is concentrated preferentially in the upper left part of the heart, giving a pink colour to it. The physiological role of this compound is like that of myoglobin in other muscular tissues (D. W. Lübbers & J. Ramírez, unpublished). Since the absorption bands of this pigment are so large in some preparations that they mask the peaks of the

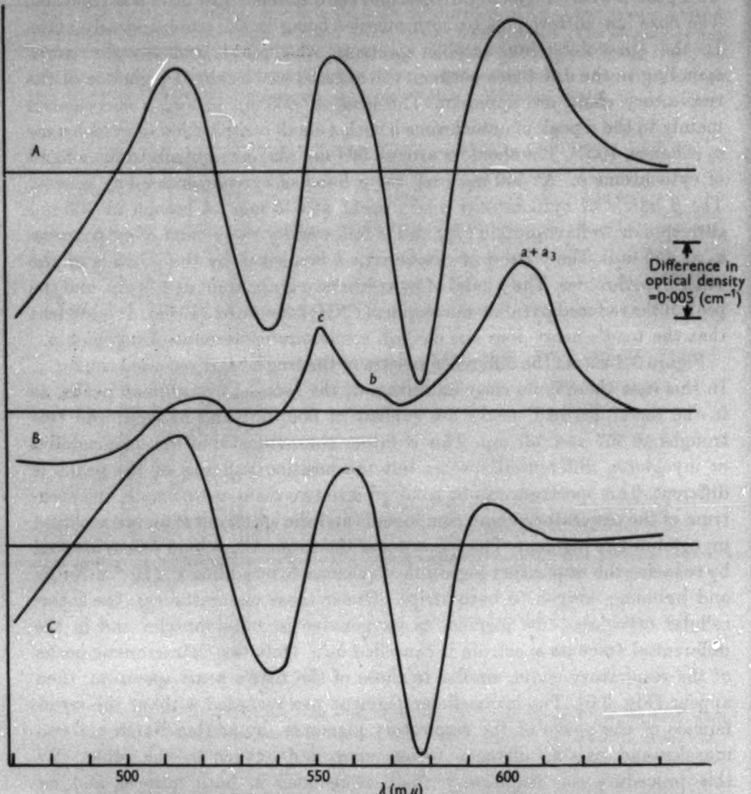


Fig. 3. Difference spectra of frog's heart strips. *A*, oxygenated minus anoxic muscle; base line, both strips in presence of oxygen. *B*, respiratory pigments reduced in cyanide-treated muscle, oxygen in both preparations. *C*, intracellular nitrogen reduced 'myoglobin-like' pigment minus the oxygenated pigment; respiratory chain reduced in both preparations with cyanide.

TABLE 1. Absorption bands in a difference spectrum (oxygenated minus reduced) of haemoglobin in frog's erythrocytes and intramuscular haemoglobin-like pigment

	Wave-length (mμ)				
	Peaks			Troughs	
Haemoglobin	597	558	512	577·5	537
Pigment	597	559	513	576–577	538

cytochromes in the reduced state, as is seen in the Fig. 3*A*, we prefer the toad's heart, which does not contain the pigment, to the frog's heart. Also, the concentration of cytochromes is about three to five times greater in the toad's heart.

By the application of the calculations of Chance (1957) to the spectroscopic data on the strips of the toad's heart it was found that the difference in concentration between the aerobic and anaerobic states of each cytochrome was equivalent to 20–45 μmoles/kg wet wt. depending on the preparation. This compares with about 10–20 μmoles/kg wet wt. in the toad's sartorius muscle (F. F. Jöbsis, personal communication) and 2–3 μmoles/kg wet wt. in the frog's sartorius muscle (Weber, 1957). The relative differential concentrations of the respiratory components, taking the concentration of cytochrome *a* as unity, are shown in Table 2. These ratios of the components are similar to those in toad's heart sarcosomes and also to the ratios in rat heart sarcosomes described by Chance & Baltscheffsky (1958). The main differences are that the cytochrome *b* is in higher concentration in the rat heart sarcosomes than in either the whole heart or heart sarcosomes of the toad. The cytochrome *c* is in a greater concentration in the heart. In toad's heart sarcosomes the ratio $a_3:a$ in 0·32 M sucrose is less than the ratio in hypotonic medium (0·086 M sucrose), which is in agreement with a similar observation in liver mitochondria (Chance & Williams, 1955*b*).

TABLE 2. Relative differential concentrations of the respiratory pigments (cytochrome $a = 1$) in toad and in rat muscle

	Toad			Rat*
	Intact heart muscle†	Heart sarcosomes		
Pigment		0·32 M sucrose	0·086 M sucrose	Heart sarcosomes
Cytochrome *a*	1	1	1	1
Cytochrome a_3	0·8	0·8	1·9	1·1
Cytochrome *b*	0·4	0·3	0·61	1·0
Cytochrome $c+c_1$	1·1	0·6	0·86	1·3
Flavoprotein	1·3	1·5	1·1	1·1
Pyridine nucleotides	5·8	—	—	4·3

* Chance & Baltscheffsky (1958). † Average of 6 muscles.

The spectral curve of the respiratory pigments in the toad heart sarcosomes is similar to that of intact muscle and is practically identical with the spectrum of rat heart sarcosomes (Chance & Baltscheffsky, 1958).

The oxidation–reduction level of cytochromes a_3, *a*, *c* and *b* in the resting aerobic steady state is about 92–98% of complete oxidation, as was shown by 2–8% further oxidation when the perfusion liquid contained either 1000–2000 μM Na amytal or 200 μM 3-hydroxy-1-heptylquinoline-*N*-oxime (HOQNO). These substances inhibit the electron flow to the cytochromes, stopping the respiration. It should be noted that whereas cytochrome a_3 is

95% oxidized in the intact muscle it is more than 99% oxidized in the isolated sarcosomes.

Changes of the respiratory components as a consequence of muscular activity. When the resting muscular strip is electrically stimulated at a constant frequency, the oxidation–reduction levels of the respiratory chain components are modified and a new steady state is reached. This state is established very rapidly; the half maximum change is reached in 2–4 contractions, which is after about 4–8 sec ($t\frac{1}{2}$ ON). Moreover, it is the same for the different components in the respiratory chain. The number of twitches is the same when different preparations are used and is independent of temperature and stimulating frequencies.

After stimulation the respiratory chain components immediately start returning to their previous oxidation–reduction level; the half time for this return ($t\frac{1}{2}$ OFF) is quite variable in different preparations. It is about 4–6 sec for cytochromes *b*, *c*, *a* and flavoprotein, but is usually 2–3 times longer in the case of cytochrome a_3 and pyridine nucleotide (see Table 3). These fast changes in the oxidation–reduction level of the respiratory chain components agree with the rapid changes in oxygen consumption (Ramírez, 1953).

The typical changes of the steady state of cytochrome *b* during the muscular activity in the frog's heart are shown in the upper trace of Fig. 4*A*. Although this heart contains a 'myoglobin-like' pigment, its interference with the cytochrome absorption changes can be eliminated by perfusing the muscle with oxygen-saturated Ringer's solution. Such a procedure always maintains the 'myoglobin-like' pigment in a fully oxidized state. The lower trace indicates the change in transmittance recorded at only one wave-length, 540 mμ, and indicates the contractions. During the muscular activity the cytochrome *b* becomes oxidized. The total change is very small. In this preparation it is 0·0017 optical density units, which represents an oxidation of 2% of the cytochrome *b*. A similar oxidation of cytochrome *b* in skeletal muscle, produced by contractions, has been described by Weber (1957), and by Jöbsis & Chance (1957).

During isolated contractions it is possible in some preparations to see changes in the cytochromes (Fig. 4*B*). These tracings show spontaneous contractions every 10 sec, and the corresponding changes in cytochrome *b*. There are very early changes in the oxidation–reduction steady state of cytochrome *b* during the contraction and a fast reduction during the resting period. Note that in this preparation the non-specific optical changes originating during the contraction are practically absent in the differential trace.

The total change reached during the contractions is a function of the stimulating frequency (Fig. 4*C*). A similar curve is obtained with the other components of the respiratory chain. At the highest frequency that the heart can contract (in these circumstances about once per second), there is still not a saturation in the cytochrome change. The limiting factor is the refractory period of the heart. The total change at the maximum is 2–6% of the total cytochrome concentration (Table 3). In skeletal muscle the changes are about 5–10%.

When muscle strips 1 mm thick are subject to stimulation at about 1 c/s at 15° C, anoxia occurs in the centre of the muscle owing to the large increase in oxygen consumption. In this case the oxygen is used up by the outer muscle fibres, leaving those in the centre without oxygen. Spectroscopically this is shown by a pronounced cytochrome reduction superimposed on the steady-state cytochrome changes due to the muscular contractions. This reduction disappears very slowly after the activity when the oxygen diffuses back again. To obviate this difficulty, stimulation frequencies of 0·5 c/s or less were used.

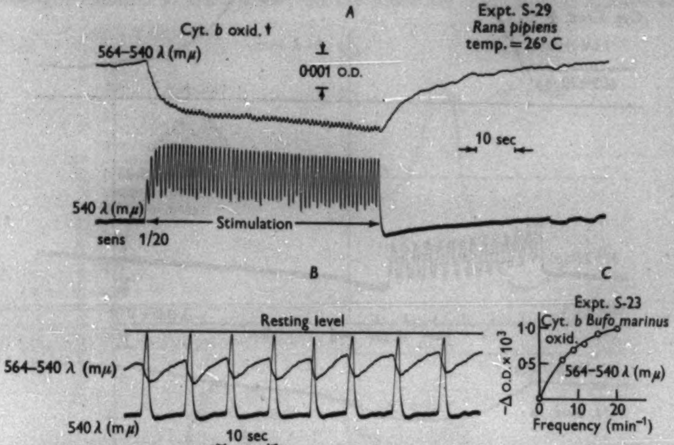


Fig. 4. Changes of cytochrome *b* produced by heart contractions in the frog. *A*, stimulation at 1 per sec: upper trace, cytochrome change recorded as the difference of absorption at 564–540 mμ; lower trace, contractions recorded by the changes of transmittance at 540 mμ. *B*, isolated contractions in the same preparation. *C*, cytochrome *b* oxidation in optical density units versus stimulation frequency in toad heart muscle.

The behaviour of the other components is similar to that of cytochrome *b*. Fig. 5*A* illustrates the reduction of cytochrome *a* during the activity, recorded at 605–620 mμ. The cytochrome *c* in most preparations becomes more reduced during the contractions but in some experiments its oxidation–reduction level did not change. The flavoprotein and pyridine nucleotide become more oxidized during activity (Fig. 5*B*). The characteristics of the cytochrome changes are about the same at temperatures between 7 and 27° C.

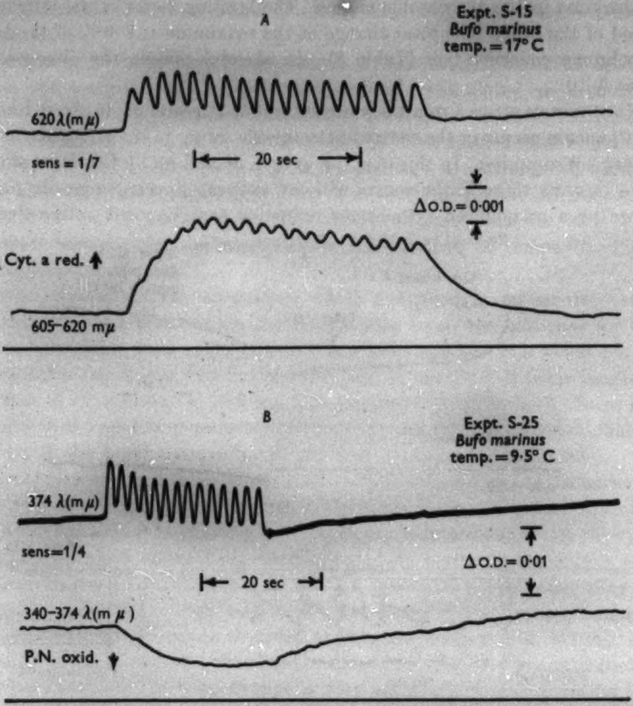


Fig. 5. Changes of cytochrome *a* (*A*) and pyridine nucleotides (*B*) as a consequence of cardiac contractions. The lower curves show the change in the respiratory pigment recorded as a difference of absorption at two wave-lengths; upper traces, changes in transmittance at one wave-length that indicates the mechanical activity. Calibration in optical density units.

TABLE 3. Typical experiment showing the changes occurring in the respiratory pigments as a consequence of cardiac activity*

Pigment	Change in optical density†	Change (μmoles/dm^3)	Total concn. (μmoles/dm^3)	Change in total concn. (%)	$t_{\frac{1}{2}}$ ON‡ sec	$t_{\frac{1}{2}}$ OFF sec
Cytochrome a_3	−0·0028	−0·51	19·3	−2·7	4	11
Cytochrome a	+0·0013	+0·83	24·4	+3·4	3–4	3·5
Cytochrome $c+c_1$	+0·00054	+0·30	26·9	+1·2	4§	6·9§
Cytochrome b	−0·0011	−0·57	9·8	−5·8	4	7·5
Flavoprotein	−0·00080	−0·77	38·5	−2·0	5	7·5
Pyridine nucleotides	−0·0052	−9·2	154·0	−6·0	5–6	12

* Stimulating frequency, 0·5/sec; temperature 20·8° C; strip thickness, 0·095 cm.
† +reduction; −oxidation.
‡ $t_{\frac{1}{2}}$ ON, etc., see text.
§ Subject to some uncertainty because of the small change in optical density.

The oxidation of pyridine nucleotide during the contractions in skeletal muscle has been reported by Connelly & Chance (1954). The changes in the various components are summarized in Table 3, and Fig. 8.

Action spectra

As a further step in the identification of the respiratory chain components participating in these reactions, similar observations were repeated at various wave-lengths in a double-beam spectrophotometer, maintaining the reference wave-length constant throughout the measurements. The optical density changes reached at the activity steady state are then plotted against the wave-length and an 'action spectrum' is obtained indicating at what wave-lengths the maxima and minima occur (Fig. 6). During the contractions a maximum absorption change occurs around 470 mμ, indicating an oxidation of flavoprotein. There is a maximum at 550 mμ and a minimum at 564–565 mμ. The latter is not very sharp, possibly because the non-specific changes in the absorption are relatively larger in that region. These changes indicate a reduction of cytochrome *c* and an oxidation of cytochrome *b*, respectively. The

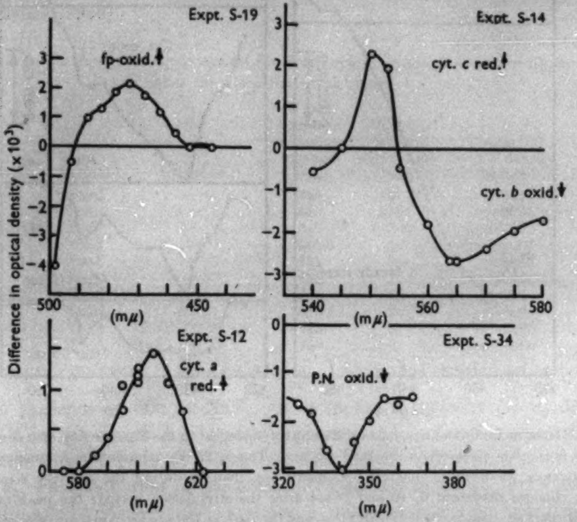


Fig. 6. Maximum change in optical density at different wave-lengths produced by the contraction in the heart (action spectra). Upper curves: left, demonstrates an oxidation of flavoproteins; right, shows a reduction of cytochrome *c* and an oxidation of cytochrome *b* during the activity. Lower curves: left, reduction of cytochrome *a*; right, oxidation of pyridine nucleotides.

oxidation of cytochrome *b* has also been observed in the Soret region (*vide infra*). There is also a reduction of cytochrome *a* with a maximum at 605 mμ and an oxidation of the pyridine nucleotides with a minimum at 340 mμ. Since all these maxima and minima correspond to the absorption peaks of the components in question, these spectra provide a good verification of the participation of the members of the respiratory chain during the contractions.

The changes in cytochrome a_3 followed at 445 mμ in muscle deserve special consideration. The action spectrum calculated from the steady-state values during the contractions does not show any distinct trough but only an irregular curve (Fig. 7*A*). If 'action spectra' are calculated from the oxidation–reduction changes at later times after the stimulation, a distinct trough around 444 mμ appears. In Fig. 7*B* this trough is already apparent at 5 sec after stimulation and still remains after 16 sec. The wave-length at which the maximum optical density appears after stimulation in several experiments is tabulated in Table 4. It is between 444 and 445 mμ, and on this basis the absorption band is identified with cytochrome a_3. The extent of oxidation of cytochrome a_3 during the muscular activity is less than the total oxidation obtained by the action of an inhibitor like amytal at concentrations which stop respiration. The lack of a distinct trough during the contractions

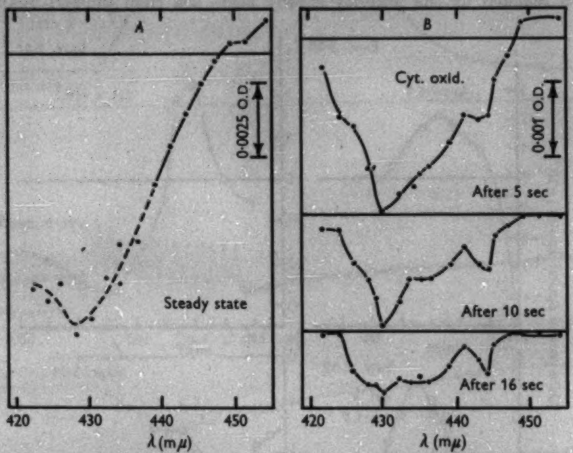


Fig. 7. Decrease in optical density at different wave-lengths in the Soret region as a consequence of muscular contractions in toad's heart. Temp. 25° C, stimulation frequency 0·5/sec, reference wave-length 455 mμ. *A*, maximum changes during the activity steady-state. *B*, changes persistent 5, 10 and 16 sec after the stimulation period; the peak at 444 mμ indicates an oxidation of cytochrome a_3 and the peak at 430 mμ an oxidation of cytochrome *b*.

is due to a great change in the light-scattering properties of the tissue at the Soret band, where changes of cytochrome a_3 have to be measured. Another factor that tends to mask the trough is the oxidation of cytochrome *b*, which has a maximum absorption at 430 mμ close to the peak of cytochrome a_3. That the return to the resting steady state of cytochrome *b* after the activity is faster than the return of cytochrome a_3 is readily observable from Fig. 7*B*. The appearance of the oxidized trough at 445 mμ in the 'action spectrum' can be seen even at the steady-state level during contractions if ~ 10 μM antimycin *A* (5·4 mg/l.) or 20 μM-HOQNO is present (Ramírez, 1958). These inhibitors decrease the extent of cytochrome *b* oxidation during activity by partially blocking the chain between cytochrome *b* and *c* (Chance, 1956).

TABLE 4. Maximum change in optical density of cytochrome a_3 in the Soret region after the stimulation of the heart

Expt.	Animal	Inhibitor	Wave-length of maximum change (mμ)	Time after stimulation (sec)	Change in optical density units*
S-22	Toad	—	445	2	−0·001
S-23	Toad	—	445	12·5	−0·0003
S-25	Toad	—	445	2·5	−0·0012
S-27	Toad	—	445·5	2·5	−0·0002
S-39	Toad	—	444	6·5	−0·0008
S-52	Frog	—	445	2	−0·0005
S-25	Toad	*A.A*†	445	2·5	−0·006
S-27	Toad	*A.A*	444·5	2·5	−0·002
S-30	Toad	HOQNO‡ 20 μM	445	3	−0·0005
S-21	Toad	NaN_3 100 μM	444	0	+0·0006

* + reduction, − oxidation. † 10 μM antimycin *A*. ‡ 3-hydroxy-1-heptyl-quinoline-N-oxime.

In the presence of 100 μM-NaN_3, the response of cytochrome a_3 during activity is in the direction of reduction rather than oxidation, and in the 'action spectrum' a peak at 445 mμ appears indicating a reduction of cytochrome a_3. This reduction in the presence of azide was shown previously in skeletal muscle by Weber (1957) and also in mitochondria from liver (Chance & Williams, 1956*b*).

Response of the toad's heart sarcosomes to the addition of ADP

Since the behaviour of the toad heart sarcosomes is similar in detail to that of rat heart sarcosomes described by Chance & Baltscheffsky (1958), it will be described only briefly. The sarcosome suspension in an oxygenated Mg^{2+}-free medium (M: sucrose 0·32, Na ethylene diamine tetraacetate 0·001, KCl 0·01, phosphate buffer 0·016, pH 7·4), in the presence of 1–3 mM-Na α-ketoglutarate or Na succinate as substrate, presents a slow respiration. Upon addition of 30–300 μM-ADP the oxygen consumption increases until the ADP is phosphorylated (respiratory control). During this increase cyclic changes of the respiratory components occur; namely pyridine nucleotides, flavoprotein and cytochrome *b* become oxidized while cytochromes *c* and *a* become reduced. Thus we see that the direction of the changes in the components are the same as those observed in the intact muscle during the contractions. Cytochrome a_3, however, does not change or shows a very small reduction (less than 0·3% of the total concentration) in the sarcosomes, although it shows over a 2% oxidation in the intact muscle. High P:O ratios around 3·7 were obtained with the sarcosome preparations when α-ketoglutarate was used as substrate.

When creatine alone or in the presence of ATP was added to the sarcosome preparation, no changes in the spectra of the cytochromes were observed.

DISCUSSION

Changes in respiratory-chain components and ATP hydrolysis

The similarity of the changes of the respiratory-chain components in heart muscle upon activity and in the sarcosomes, on addition of ADP, strongly suggests the view that ADP is released during the muscular contractions, However, our present data do not allow us to make quantitative comparisons of the energy released by ATP hydrolysis and the total energy expenditure, since the work performed by the heart and the changes in heat production were not measured.

In our conditions the accurate time relationship between contraction and cytochrome changes is very difficult to establish because of the impossibility of completely eliminating the interference of the non-specific optical changes originating during the activity. Nevertheless, we can say that the cytochrome change begins during the contraction phase of the heart twitch.

Comparison with skeletal muscle

The fast changes in the steady-state level of the cytochromes when muscular activity starts and stops (Table 3) indicate a very rapid mechanism of control and interaction between the contractile systems and the energy-producing mechanism of the respiratory chain. This is in contrast to skeletal muscle, where three times as many twitches are needed to reach half the maximum oxidation–reduction change of the respiratory pigments (Chance & Connelly, 1957) and the cytochrome changes persist several minutes after a short tetanus. In fact, the resting steady state in sartorius muscle is reached in some cases after 20–30 min (Weber, 1957; F. F. Jöbsis, personal communication).

There are several factors which might be responsible for the difference in behaviour between cardiac and skeletal muscle.

(1) There are a large number of sarcosomes contained in heart muscle (Weinstein, 1954; Harman, 1956; Sjöstrand, Andersson-Cedergren & Dewey,

1958). The sarcosome size is small, *ca.* 0·8 μ, and they exist along practically every fibril. This morphological arrangement greatly decreases the diffusion time between the fibrils and sarcosomes, thus increasing the synchronization of the sarcosome response with the fibril activity. In the frog's sartorius muscle, however, the sarcosomes are bigger, 10×1 μ, and are rather widely spaced in the muscle, having several fibrils between them. This difference in the number of sarcosomes between skeletal and cardiac muscle is in agreement with the fact that the equivalent concentration of cytochromes is greater in the heart.

(2) There is a low concentration of creatine phosphate (CP) in the cardiac muscle as compared with the five to ten times higher concentration found in skeletal muscle (Clark, Eggleton, Eggleton, Gaddie & Stewart, 1938). Furthermore, in the cardiac muscle the ATP–creatine transphosphorylase activity is six times lower (Oliver, 1955). Hence the competition of the respiratory chain and the ATP–creatine transphosphorylase in the rephosphorylation of ADP is probably not so important in the heart and is in favour of the respiratory chain. In the skeletal muscle, when ADP is produced as a consequence of muscular activity, the ATP–creatine transphosphorylase system could rephosphorylate ADP to ATP and produce creatine (C). Upon cessation of the activity, when the ADP production stops its concentration decreases owing to the action of the respiratory chain. Then the equilibrium of the reaction

$$\mathrm{CP} + \mathrm{ADP} \rightleftharpoons \mathrm{ATP} + \mathrm{C}$$

catalysed by the ATP–creatine transphosphorylase, is reversed, that is displaced to the left, producing CP and ADP. This ADP interacts with the respiratory chain, where it is finally rephosphorylated. As a consequence of this interaction between the transphosphorylase system and the respiratory chain in the skeletal muscle, the cytochrome changes and the oxygen consumption are prolonged in time.

Moreover, there is some evidence that besides creatine phosphate, the phosphate compounds of carnosine and anserine (Severin & Meshkova, 1953; Meshkova & Zajieva, 1953; Nagradova, 1956) can also rephosphorylate the ADP to ATP in the skeletal muscle whereas the cardiac muscle is devoid of the carnosine–anserine fraction (Schmidt & Cubiles, 1955).

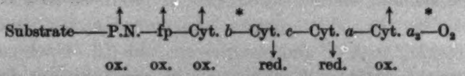
(3) The effect of variations in temperature on the cytochrome changes during the activity in cardiac and skeletal muscle is also different. In the heart muscle cytochrome kinetics between 7 and 27° C are relatively constant. In frog skeletal muscle the amplitude of the cytochrome change decreases with increasing temperature and almost reaches zero at 25° C. (B. Chance, personal communication). As with cardiac muscle, the cytochrome changes in isolated sarcosomes are about the same at different temperatures (Chance & Sacktor, 1958). These data may mean that in skeletal muscle extra-sarcosomal ATP

phosphorylating systems which are temperature-dependent are present, but in cardiac muscle these systems are not active.

All these observations indicate that in the cardiac muscle the main source of resynthesis of ATP is the respiratory chain, and therefore the coupling between the contractile mechanism and the cytochrome system is 'tight'. In the skeletal muscle other systems (creatine, carnosine, anserine) can suddenly produce a high amount of ATP, and in the end the respiratory, or glycolytic, mechanisms 'recharge' these systems and the cytochrome chain in these circumstances works over a considerable period of time to fulfil the instantaneous high energy requirements of the muscle.

Crossover points

When the condition exists between two adjacent components of the respiratory chain such that upon increase of the respiratory rate there is an oxidation of the component on the substrate side and a reduction of the component on the oxygen side, then the point between these two components is called a 'crossover point' (Chance & Williams, 1956*b*). In the intact heart two crossover points in the respiratory chain are found when the electron flow increases upon mechanical activity (Fig. 8). One point is between cytochromes *b* and *c* and the other between cytochrome a_3 and oxygen. In the sarcosomes, upon addition of ADP only one crossover point was identified and that was between cytochromes *b* and *c*. According to the crossover theorem of Chance & Williams (1956*b*), these points indicate sites of phosphorylation, although it is possible for a site of phosphorylation to be located at a place where no crossover appears.



$$\text{Substrate}\text{——}\overset{\uparrow}{\underset{\text{ox.}}{\text{P.N.}}}\text{—}\overset{\uparrow}{\underset{\text{ox.}}{\text{fp}}}\text{—}\overset{\uparrow}{\underset{\text{ox.}}{\text{Cyt. }b}}\overset{*}{\text{—}}\underset{\substack{\downarrow\\ \text{red.}}}{\text{Cyt. }c}\text{—}\underset{\substack{\downarrow\\ \text{red.}}}{\text{Cyt. }a}\text{—}\overset{\uparrow}{\underset{\text{ox.}}{\text{Cyt. }a_3}}\overset{*}{\text{—}}O_2$$

Fig. 8. The respiratory chain. The upward arrows indicate an oxidation, the downward arrows a reduction of the components during the heart activity. The stars signify the crossover points.

In heart muscle NaN_3 at low concentrations changes the response of cytochrome a_3 during the muscular activity from an oxidation to a reduction which makes the crossover point between cytochrome a_3 and oxygen disappear. A possible explanation of this effect is that the NaN_3 prevents the phosphorylation between cytochrome a_3 and oxygen, since it is known that 10^{-4} M azide decreases the phosphorylation efficiency, that is, the P:O ratio in heart sarcosomes with α-ketoglutarate as substrate (Slater, 1955). However, Chance & Williams (1956*b*) found no decrease in P:O ratio in liver mitochondria oxidizing succinate in the presence of $2{\cdot}76 \times 10^{-4}$ M-NaN_3 and Loomis & Lipmann (1949) found a decrease in P:2Fe $(CN)_6^{4-}$ ratio only with 1×10^{-3} M azide, in kidney homogenates. If the phosphorylation between cytochrome a_3 and oxygen exists, then intermediates involved in the reaction are apparently labile since in sarcosomes isolated from toad's heart, in a procedure which gives respiratory control and the usual values of the P:O ratio, no crossover point between cytochrome a_3 and oxygen is found. In addition, it has not been possible to obtain a consistent demonstration of the oxidation of cytochrome a_3 in other types of muscle (toad sartorius, frog sartorius and turtle neck muscle, F. F. Jöbsis, personal communication.) The cardiac muscle could therefore have a uniquely high P:O ratio (i.e. around 4·7 with a substrate like α-ketoglutarate) if the additional phosphorylation site of the cytochrome a_3 level is taken into consideration, since in the isolated sarcosomes the P:O ratio is approximately 3·7. Work is in progress to confirm this point in a more direct way.

The spectroscopically observed oxidation of cytochrome a_3 is not contradictory to the fact that an increase in respiration rate is accompanied by an increase in the concentration of reduced cytochrome a_3 (see Appendix).

SUMMARY

1. The components of the respiratory chain in the intact cardiac muscle of the toad and of the frog have been identified by means of spectrophotometric techniques. The relative concentrations are similar to those in toad heart sarcosomes. The concentrations are 2–3 times higher than in the toad's sartorius muscle.

2. The oxidation–reduction level of the cytochromes in the resting state, including cytochrome a_3, is 92–98% of oxidation.

3. During mechanical activity the oxidation–reduction steady-state levels of the respiratory chain components change very rapidly; the time for reaching half the maximum change is attained during 2–4 contractions. The maximum change is nevertheless very small, about 2–6% of the total concentration. These characteristics are approximately the same for the different components.

4. 'Action spectra' of the heart muscle taken during the contractions show optical absorption changes having maxima and minima corresponding to the peaks of the respiratory-chain components. By this procedure it was shown that pyridine nucleotides, flavoprotein, and cytochromes b and a_3 are oxidized during activity and cytochromes c and a are reduced. In some preparations the cytochrome c does not change.

5. The changes of the respiratory-chain components in the sarcosomes on addition of ADP are the same as in contracting muscle. This is in favour of the idea that ADP is released during muscular activity.

6. The finding that cytochrome a_3 does not change on addition of ADP to sarcosomes suggests that there is an additional phosphorylation site in the intact heart muscle, which has not been demonstrated in intact skeletal muscle or in isolated mitochondria.

7. The fast changes during activity of the respiratory components in

cardiac muscle, as compared with skeletal muscle, are interpreted in terms of higher concentration of cytochromes, greater number and smaller size of sarcosomes and lower concentration of creatine phosphate, ATP–creatine transphosphorylase and similar ATP regenerating systems.

8. It is concluded that in the heart the respiratory chain is the main source of ATP resynthesis.

9. The observed oxidation of cytochrome a_3 is compatible with the idea that in order to increase the oxygen consumption the concentration of reduced cytochrome a_3 must increase.

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APPENDIX

The unusual oxidation of cytochrome a_3 during heart-muscle activity can be shown to be consistent with the fundamental equation of oxygen consumption in a cytochrome oxidase system (Chance, Williams, Holmes & Higgins, 1955).

$$-\frac{d[O_2]}{dt} = k_1[a_3''][O_2].$$

The equation states that the oxygen consumption is proportional to the concentration of reduced cytochrome a_3, $[a_3'']$, and to the oxygen concentration $[O_2]$. Since oxygen concentration is constant in our experiments, the cytochrome a_3'' concentration must be increased during the activity in order to explain the increase in oxygen consumption. At first glance this might seem to be in contradiction to the observed cytochrome a_3 oxidation. However, if a crossover point between cytochrome a_3 and oxygen does imply that there is a phosphorylation at this level, we may postulate the existence of a compound $a_3'' \sim I$, the inhibited form of a_3'', that stores energy in the resting state. It is assumed that spectroscopically there is no difference between a_3'' and $a_3'' \sim I$ (Chance & Williams, 1956*a*), and $a_3'' \sim I$ does not combine with oxygen. During the muscular activity $a_3'' \sim I$ breaks down, owing to the increase in ADP concentration, and therefore the concentration of free a_3'' increases; in consequence the rate of oxygen consumption is raised, as is required by the above equation. Part of the free a_3'' is oxidized, as the spectrophotometric data indicate, but the ratio in these circumstances

$$\frac{\text{free } [a_3''] \text{ (activity)}}{\text{free } [a_3''] \text{ (rest)}} > 1,$$

and the respiratory increase is proportional to the newly released a_3''.

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CORTICOSTEROID SECRETION BY THE AUTOTRANSPLANTED ADRENAL GLAND OF THE CONSCIOUS SHEEP

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The role of a particular organ in a complex regulatory process cannot be clearly understood unless direct observations on its function can be made, without at the same time disturbing other components of the process. This has not previously been possible in the study of adrenal function, because the gland is accessible only by surgical exposure, and this procedure inevitably causes changes in adrenal secretory activity which may seriously affect the interpretation of experimental findings. Indirect methods of assessing adrenal secretory activity give only a crude index, and the precision of recently developed methods for adrenal hormone assay cannot be completely utilized.

One attempt to overcome this difficulty has been to collect adrenal venous blood from conscious experimental animals of suitable size, such as the dog, either by direct cannulation after previous 'de-afferentation' of the region (Satake, Suguwara & Watanabe, 1927) or by previous implantation of a cannula into the adrenal vein (Hume & Nelson, 1954; Egdahl & Richards, 1956). The former procedure requires exceptional surgical skill and careful training of the animal, and the second suffers from the disadvantage that the useful period after implantation of the cannula is severely limited, so that collections must be made before the processes of healing are completed. Neither method permits long-term serial observations, nor do they permit experimental alterations in the local environment of the gland.

Another method is to transplant one adrenal gland complete with its blood vessels to a superficial site, and to study its function after the animal has recovered from the surgical intervention. A technique for transplanting the adrenal gland of the sheep to a combined carotid-artery–jugular-vein skin loop has been described elsewhere (McDonald, Goding & Wright, 1958). This preparation has the advantage that both the adrenal artery and vein are accessible for experimental purposes in the conscious unrestrained animal. The value of such a preparation would depend on the adequate demonstration of normal function with respect to the process being investigated. It has already been shown that successful transplants of this type are histologically indistinguishable from normal (Levy & Blalock, 1939; McDonald *et al.* 1958), while their secretion is sufficient to maintain life and to permit the normal regulation of electrolyte balance under the stress of Na^+ depletion.

This paper is concerned with the investigation of corticosteroid secretion by such transplanted adrenal glands, with reference to the nature of the individual corticosteroids and their normal secretion in the conscious unrestrained state, the response to adrenocorticotrophic hormone, and the effect of Na^+ depletion. The findings indicate that the autotransplanted adrenal gland is a suitable preparation for the investigation of adrenal function in the sheep.

METHODS

Two sheep with adrenal transplants (Tr. 1 and Tr. 4) were used in this study. Details of the operative procedures and operation histories of the animals have been published elsewhere (McDonald *et al.* 1958). In each sheep the left adrenal gland was transplanted to a combined carotid-artery–jugular-vein skin loop so that it received its arterial blood from the carotid artery and drained its venous blood into the jugular vein. At subsequent operations the right adrenal gland was removed, the left carotid sinus denervated, and a right parotid fistula and carotid-artery loop were constructed.

The sheep were maintained on a diet of mixed oaten and lucerne chaff, giving average Na^+ and K^+ intakes from this source in the range of approximately 100 and 300 m-equiv/day respectively. Loss of Na^+ from the parotid fistula was made good by the daily intrarumenal administration of 25–50 g $NaHCO_3$, or by self-selection of 2·5% $NaHCO_3$ solution from a special bin attached to the cage. It was noted that after the self-selection regime was begun, the sheep ate approximately twice as much as before, gained weight, and were generally in much better condition.

If the supplementary $NaHCO_3$ intake was stopped, the sheep rapidly developed a Na^+ deficit of 400–600 m-equiv in 3–4 days. The relationship between the salivary $Na^+:K^+$ ratio and Na^+ deficit, described by Denton (1957), was also demonstrated in these sheep, and Na^+ depletion was designated as 'moderate' if the salivary $Na^+:K^+$ ratio indicated a deficit of 200–400 m-equiv Na^+, and 'severe' if the salivary $Na^+:K^+$ ratio indicated a deficit of 400–600 m-equiv Na^+.

Method of obtaining adrenal venous blood

The animal was first restrained on a table so that a polythene cannula with 2 mm bore could be inserted through a needle into the 'adreno-jugular' vein cranial to the transplant. The tip was manoeuvred to lie opposite the adreno-jugular anastomosis, and the cannula was fixed in position with a few skin sutures. The cannula was then washed out with heparinized saline, filled with a solution of heparin 1000 i.u./ml. saline, and plugged. If intravenous injections were to be made in the subsequent experiment, a second polythene tube was inserted into the right jugular vein and fixed in place. A pneumatic cuff was applied loosely round the skin loop cranial to the transplant so that when inflated it would occlude the jugular vein. The sheep was then returned to its cage and left undisturbed with access to food and water for at least two hours.

To collect adrenal venous blood, the animal was first heparinized with an intravenous injection of heparin 10,000 i.u. via one of the polythene cannulae. The pneumatic cuff was then inflated gently to a pressure of approximately 200 mg Hg to ensure that the vein was compressed tightly around the cannula, the plug was removed from the cannula and the jugular vein was lightly occluded on the cardiac side of the transplant by a piece of sponge rubber clipped around the loop. This procedure did not appear to disturb the sheep, which were well adapted to minor interference of this nature.

After allowing approximately 10 ml. of adrenal venous blood to wash out the cannula, and making sure there was no obstruction to the arterial or venous blood flow, the adrenal venous blood was collected for analysis. The length of the polythene tube was approximately 3 ft (91 cm), and the collecting end was held approximately 18 in. below the level of the transplant. The rate at which heparinized sheep's blood siphoned through similar tubing of the same length, with the outlet held 18 in. below the container, indicated that, up to rates of 6–7 ml./min, there was no obstruction to blood flow. In later experiments the length of fine bore tubing was limited to 12 in, and connected to an extension tube with a bore of 4 mm. This permitted higher rates of blood flow without increase in adrenal venous pressure; but use of either method on one sheep (Tr. 1), whose adrenal blood flow was less than 7 ml./min, showed that there was no significant difference in rates of blood flow or corticosteroid secretion. In one sheep with an adrenal blood flow of more than 7 ml./min, the adreno-jugular vein became distended when the first collecting method was used. This prompted the adoption of the second method. At the end of the period of collection the occluding cuff on the cardiac side of the transplant was released, the cannula was washed free from blood with heparinized saline and replugged. The pneumatic cuff was then deflated until the next collection period.

Corticosteroid analysis

The adrenal venous blood was collected over a timed interval into graduated cylinders, protected from light and immersed in crushed ice. After measuring the volume, a small portion was taken for haematocrit determination, and the remainder was immediately centrifuged. The separated plasma was stored during the night in a refrigerator, and 10–15 ml. of each sample was extracted the following day. Losses occurring during the extraction and chromatographic procedures were ascertained by the addition of 4-^{14}C-labelled cortisol and corticosterone to each sample and measurement of the recovered radioactivity at the final stage of analysis. Thus all figures for corticosteroid secretion refer to the extractable plasma corticosteroids. A modified chromatographic procedure developed in this laboratory was used to identify and measure the individual corticosteroids (Reich, 1958). The rates of secretion of the corticosteroids were calculated from the adrenal plasma flow and the concentration of adrenal venous plasma corticosteroid. No correction was made for concentration of circulating plasma corticosteroid, because of the relatively low levels, and, principally, the uncertainty of the degree to which they were metabolized during their passage through the gland.

Drugs

Adrenocorticotrophic hormone, ACTH, 'Corticotrophin' (Commonwealth Serum Laboratories, Melbourne, Australia), Batch No. P. 116, prepared from hog pituitary by the process of Payne, Raben & Astwood (1950), without purification on oxycellulose, and assayed at 1·0 i.u./mg by the Munson modification of the original Sayers assay (Gray & Munson, 1951). This was dissolved in 0·85% NaCl solution for intravenous injections.

Heparin. Boots Pure Drug Co., Nottingham, England.

Radioactive corticosteroids. Samples of 4-^{14}C-labelled cortisol and corticosterone were supplied through the Endocrinology Division, National Institutes for Health, U.S. Public Health Service, U.S.A.

Injection apparatus. A Palmer slow injector was used for the intravenous injection of ACTH.

Experimental procedure

Aseptic technique was used throughout. The polythene cannulae were sterilized by heating to 60° C in a solution of chloromycetin and sulphathiazole for 30 min and then washed out with sterile saline. Control samples were taken at least 2 hr after any disturbance to the animal, and 1½–2½ hr after ACTH injections.

The loss of blood due to sampling adrenal venous blood was 200–250 ml. over a period of 5–6 hr. Adrenal venous blood used for washing out the cannula was collected into a sterile container and injected back into the sheep at the end of the experiment. After removal of the cannulae, the sheep were given intramuscular injections of penicillin 1 Mu., streptomycin 0·5 g, and 5 ml. of a colloidal iron preparation ('Imferron', Benger Laboratories, Cheshire, England). The minimum interval between experiments on any one sheep was 3 weeks, and no troubles were encountered from the cannulation procedure.

RESULTS

The Δ^4-3 ketosteroids detected in the adrenal venous plasma of the conscious sheep were cortisol, corticosterone, cortisone, aldosterone and an unidentified substance X (Fig. 1). ('Substance X' had a mobility slightly less than corticosterone when chromatographed in toluene: 75% methanol; it gave a positive yellow soda fluorescence, a positive blue tetrazolium reaction and a negative Zimmerman reaction.) The main constituent was cortisol, the secretion of

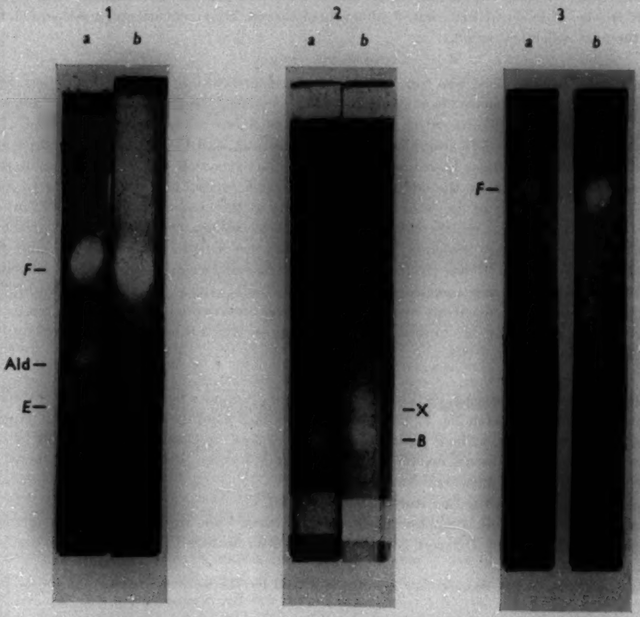

Fig. 1. Corticosteroids in venous blood from transplanted adrenal gland (Tr. 1). After chromatographic separation the paper strips were treated with 2 N-NaOH and photographed in strong ultra-violet light, using Ilford PanF film and a Wratten G. 15 filter. *a*, Reference strip; *b*, experimental strip. 1. *F* = cortisol; Ald. = aldosterone; *E* = cortisone. Eluate from first chromatogram run for 9½ hr in toluene: 75% methanol at 30° C. 2. X = unidentified Δ^4-3-ketosteroid; *B* = corticosterone. Eluate from first chromatogram run for 2½ hr in toluene: 75% methanol at 30° C. 3. *F* = cortisol. Portion of the eluate from first chromatogram run for 2½ hr in toluene: 75% methanol at 30° C.

the other steroids in significant amounts being dependent on stimulation by ACTH or sodium depletion. In the analysis of samples the main emphasis for quantitative estimation was concentrated on cortisol, corticosterone and aldosterone. The presence of cortisone and 'substance X' was always noted but they were not always estimated quantitatively.

Corticosteroid secretion of the non-stimulated adrenal transplant in the conscious sheep

The blood samples were obtained while the animal seemed to be in its normal state (standing quietly in its cage). A period of at least 2 hr after the animal had been handled for any reason elapsed before any collection was made.

Not Na^+-depleted. A total of nineteen samples of adrenal venous blood obtained from the two sheep were analysed. Included among these were five consecutive samples from Tr. 1, and four consecutive samples from Tr. 4, obtained as controls for other experiments, and five samples obtained 90–180 min after the end of a period of ACTH stimulation.

The main corticosteroid present was cortisol. Its secretion rate was very variable, the range being 0·04–0·60 mg/hr, with a mean secretion rate of 0·21 mg/hr ±0·05 S.E. for Tr. 1 (10 samples); and 0·06–0·56 mg/hr with a mean secretion rate of 0·37 mg/hr ±0·06 S.E. for Tr. 4 (9 samples). The difference between the two sheep was not significant, because of the high degree of variance. Corticosterone was present in a much smaller amount, the rate of secretion again varying greatly in the two sheep. In four samples from Tr. 1 the secretion rate ranged from 0·003 to 0·016 mg/hr, but it was usually not detectable in 15 ml. of plasma, and on one occasion could not be detected in 45 ml. of pooled plasma. In six samples from Tr. 4 the secretion rate of corticosterone ranged from 0·001 to 0·010 mg/hr, and in three samples it was not detected in the 15 ml. plasma analysed. Aldosterone was detected in only one sample obtained from Tr. 4 (secretion rate 0·004 mg/hr). On this occasion the sheep's salivary $Na^+:K^+$ ratio indicated a moderate degree of Na^+ depletion 8 hr before the experiment. At this time the Na^+ deficit was repaired by the intrarumenal administration of 300 m-equiv $NaHCO_3$ and at the time the blood sample was taken the salivary $Na^+:K^+$ ratio was normal. In the final control sample for this experiment, taken 103 min after a period of ACTH stimulation, no aldosterone was detected in 15 ml. plasma. On no other occasion was aldosterone detected in up to 75 ml. of pooled plasma samples. Cortisone was usually detected in small amounts in samples from Tr. 4, and occasionally in samples from Tr. 1. Substance X was usually not present in samples from either sheep.

Na^+-depleted. A total of thirteen samples, ten from Tr. 1 and three from Tr. 4, were analysed. Five samples from Tr. 1 and two from Tr. 4 were obtained

during moderate Na^+ depletion (salivary $Na^+:K$ = 139/29–140/33), and five samples from Tr. 1 and one from Tr. 4 during more severe Na^+ depletion (salivary $Na^+:K^+$ = 77/89–120/50). The adrenal corticosteroid secretion differed significantly in these two stages of Na^+ depletion, the range being similar for both animals. In moderate Na^+ depletion the secretion rate of cortisol ranged from 0·11 to 0·67, the mean being 0·28 mg/hr. Corticosterone ranged from less than 0·001 to 0·02, the mean secretion rate being 0·007 mg/hr. Aldosterone ranged from 0·007 to 0·021 with a mean secretion rate of 0·012 mg/hr. In severe Na^+ depletion the secretion rate of cortisol ranged from 0·33 to 1·20 with a mean secretion rate of 0·74 mg/hr. Corticosterone ranged from 0·005 to 0·073 with a mean secretion rate of 0·031 mg/hr. Aldosterone ranged from 0·006 to 0·024 with a mean secretion rate of 0·010 mg/hr. Cortisone was almost always detected, the highest secretion being about 0·010 mg/hr, while substance X was detected only in five samples, the highest rate of secretion being 0·006 mg/hr.

In summary, the corticosteroid secretion by the unstimulated sheep's adrenal transplant when in normal Na^+ balance consisted almost entirely of cortisol, which was secreted at a very variable rate within the range of 0·04–0·60 mg/hr. The characteristic feature of the adrenal corticosteroid secretion in Na^+ depletion was the appearance of aldosterone in amounts readily detectable in 10–15 ml. adrenal venous plasma. The secretion rates of cortisol and corticosterone differed significantly in different stages of Na^+ depletion, being hardly affected by moderate Na^+ depletion, but definitely increased by severe depletion. On the other hand, the secretion rate of aldosterone did not appear to be affected by the degree of depletion, the output ranging between 0·006 and 0·024 mg/hr in both stages.

Corticosteroid secretion by the ACTH-stimulated adrenal transplants

A preliminary experiment was first carried out to determine the rate of intravenous injection of ACTH necessary to stimulate the secretion of corticosteroids by the transplant. Bush & Ferguson (1953) quoted a rate of 0·3 i.u./hr of ACTH as being sufficient to stimulate the adrenal gland in their experiments on anaesthetized sheep. Using Tr. 4 in normal Na^+ balance it was found that during the continuous intravenous infusion of ACTH (in saline), at rates of 0·3 or 0·6 i.u./hr, there was no immediate increase in the output of cortisol and corticosterone. When the rate was increased to 1·2 i.u./hr there was a small but definite increase in both cortisol and corticosterone. After this experiment it was decided that rates of injection corresponding more nearly to the dose used in other species on a body-weight basis (Bush, 1952) would be satisfactory, and in all subsequent experiments the rate of ACTH injection was in the range of 5–6 i.u./hr.

Not Na^+-depleted. The findings were fairly consistent in both sheep. There was a prompt and approximately linear increase in the outputs of cortisol and corticosterone (Fig. 2, Table 1). In both sheep the ACTH injection was continued for 95–150 min. In one experiment on each sheep, in which the ACTH was injected beyond 118 min, the secretion rate of cortisol and corticosterone

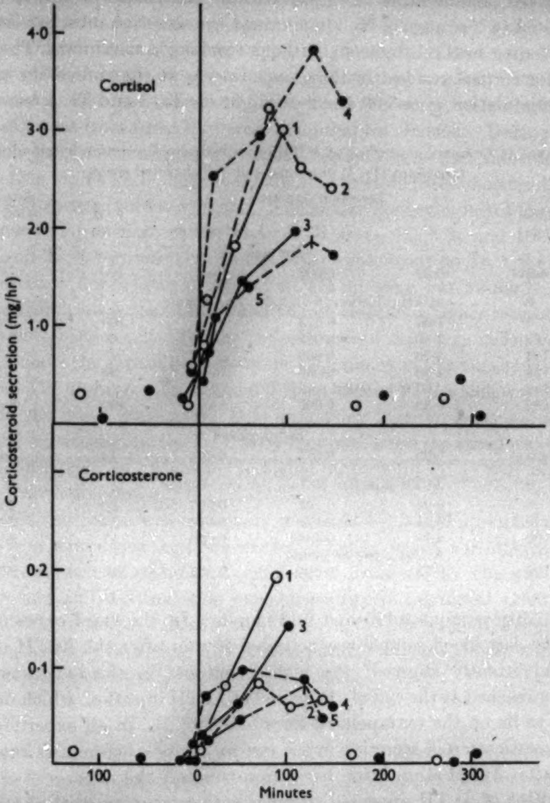

Fig. 2. Sheep not depleted of Na⁺. Effect of intravenous injection of ACTH at constant rates on corticosteroid secretion by transplanted adrenal glands. 1, Tr. 1, ACTH 6·3 i.u./hr; 2, Tr. 1, ACTH 5·6 i.u./hr (self-selection of $NaHCO_3$); 3, Tr. 4, ACTH 6·1 i.u./hr; 4, Tr. 4, ACTH 5·6 i.u./hr (self-selection of $NaHCO_3$); 5, Tr. 4, ACTH 5·2 i.u./hr. The vertical line at time 0 indicates the commencement of ACTH injection, and the lines joining the points after time 0 indicate the period of ACTH injection in each experiment. The points before time 0 and after the indicated period of ACTH injection represent corticosteroid secretion rates during the control periods.

was observed to fall despite the continued stimulation. This suggested that under the conditions of these experiments the maximum capacity of the gland to produce cortisol and corticosterone was reached, being 3·3 and 3·96 mg/hr of cortisol and 0·083 and 0·097 mg/hr of corticosterone for Tr. 1 and Tr. 4 respectively. In the other three experiments the period of ACTH injection was limited to the range of 95–110 min, and the secretion rates of cortisol and corticosterone rose continuously without reaching a maximum. The highest values for cortisol reached in these experiments at the end of the period of ACTH stimulation were 3·09 and 2·04 mg/hr for Tr. 1 and Tr. 4 respectively, and for corticosterone 0·195 and 0·142 mg/hr. In the third experiment on Tr. 4 the last blood sample was collected 25 min after the ACTH injection was inadvertently stopped: the highest outputs in this experiment were probably reached at the end of the period of ACTH injection, which one could assume to lie on the extrapolated lines (see Fig. 2). In all experiments the rates of corticosteroid secretion in the control periods before and more than 85 min after ACTH stimulation were approximately the same.

TABLE 1. Comparison of effect of ACTH on corticosteroid secretion by an adrenal transplant (Tr. 1) in normal and Na⁺-depleted states

Time (min)	Secretion (mg/hr)			$F:B$ ratio	Adrenal blood flow (ml./min)
	F	B	Aldosterone		
		Normal			
Control	0·60	0·006	—	100	7·5
0	ACTH injection (5·6 i.u./hr. I.V.) begun				
7	1·30	0·013	—	100	7·4
68	3·30	0·083	—	40	8·0
104	2·70	0·059	—	46	8·7
138	2·50	0·066	—	38	8·5
142	ACTH injection ended				
262	0·30	0·003	—	100	8·5
		Na⁺-depleted			
Control	0·99	0·073	0·008	14	3·01
0	ACTH injection (5·3 i.u./hr. I.V.) begun				
8	2·90	0·147	0·007	20	2·93
50	3·04	0·178	0·015	17	2·78
90	2·75	0·166	0·020	17	2·80
100	ACTH injection ended				
190	0·73	0·005	0·007	146	2·78

The effect of ACTH appeared to differ with respect to cortisol and corticosterone outputs according to whether the sheep were maintained on the self-selection regime or not. In the latter case cortisol secretion rose to a greater extent than in the former over a comparable period of stimulation, whereas the reverse held for corticosterone (Fig. 2). In one experiment on Tr. 1, aldosterone appeared in trace amounts after 1 hr of ACTH stimulation and increased a little 30 min later, but none was detected in the last control sample. No aldosterone was detected in the other experiments in 45 ml. samples of pooled plasma obtained during ACTH stimulation.

The secretion rates of both cortisone and 'substance X' increased during ACTH stimulation. The maximum rate of ACTH-stimulated secretion of cortisone averaged approximately 0·012 mg/hr, compared with the average non-stimulated rate of secretion of approximately 0·002 mg/hr. 'Substance X', even when initially absent, always appeared during ACTH stimulation, the maximum measured rate of secretion being about 0·015 mg/hr.

Na^+*-depleted.* The response to ACTH when the sheep were depleted of Na^+ was different from when they were in normal Na^+ balance. During ACTH stimulation the secretion rates of cortisol, corticosterone and aldosterone all increased (Fig. 3; Table 1), but the maximum secretion rates of cortisol, which were reached earlier (between 60 and 75 min after the beginning of the ACTH injection) were lower than in the undepleted sheep, i.e. 3·04 and 1·78 mg/hr for Tr. 1 and Tr. 4 respectively. In one other experiment on Tr. 1 the rate of cortisol secretion 5 min after the end of ACTH injection was 2·0 mg/hr, which was only slightly lower than that found 73 min before. A higher rate of secretion may have been reached for the period between these two collections. In each experiment the rate of corticosterone secretion rose to a maximum, being 0·178 and 0·112 mg/hr for Tr. 1, and 0·078 mg/hr for Tr. 4, which were similar to those in the undepleted state. It is interesting that the lower outputs for cortisol and corticosterone (in Tr. 4) were obtained when the sheep was in only moderate Na^+ depletion, whereas the higher outputs (in Tr. 1) were obtained in severe Na^+ depletion.

Aldosterone secretion was invariably increased by ACTH stimulation. The highest rate of stimulated secretion was 0·025 mg/hr, which was little different from the lowest rate of stimulated secretion of 0·020 mg/hr. The amount of increase during ACTH stimulation varied according to the initial value, being relatively little when the initial output was high. The rate of secretion of aldosterone, initially or during ACTH stimulation, was not closely related to the rates of secretion of other corticosteroids (Fig. 3; Table 1).

Control experiments

Four or five consecutive blood samples were collected at intervals of 30 min–1 hr from the Na^+-depleted and undepleted sheep. The variation of individual corticosteroids fell into a fairly narrow range for each experiment, aldosterone being the most constant.

In each of three experiments on Tr. 1 and Tr. 4 in Na^+-undepleted state, the corticosteroid secretion rates were respectively: cortisol, (Tr. 1) 0·038–0·165 mg/hr, 0·150–0·382 mg/hr, and (Tr. 4) 0·017–0·127 mg/hr; corticosterone, (Tr. 1) none detected in 45 ml. pooled plasma, 0·007 mg/hr (average of 60 ml. pooled plasma samples) and (Tr. 4) 0·009 mg/hr (average of 60 ml. pooled plasma samples). No aldosterone was detected in 60–75 ml. of pooled plasma samples in any of these experiments.

In one experiment on Tr. 1, when moderately Na⁺-depleted, the range of cortisol secretion was 0·103–0·330 mg/hr, corticosterone secretion 0·002–0·010 mg/hr and aldosterone 0·010–0·013 mg/hr.

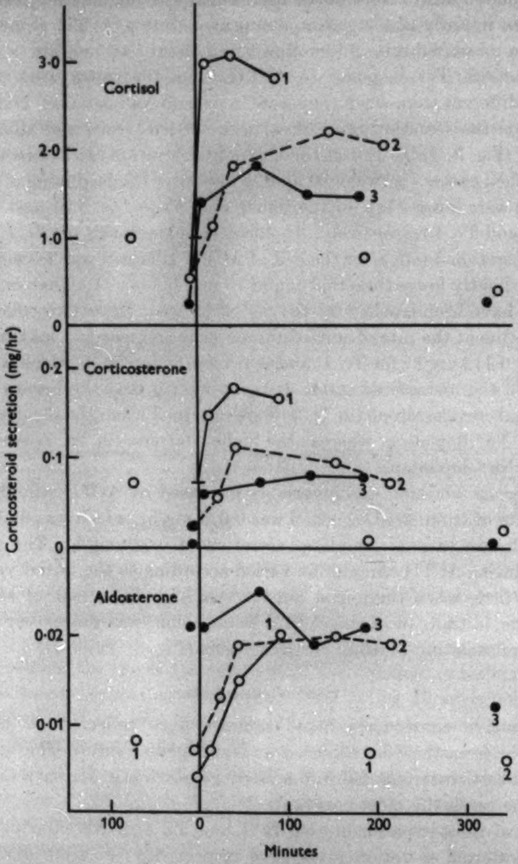

Fig. 3. Sheep depleted of Na⁺. Effect of intravenous injection of ACTH at constant rates on corticosteroid secretion by transplanted adrenal glands. 1, Tr. 1, ACTH 5·3 i.u./hr, severely depleted of Na⁺; 2, Tr. 1, ACTH 5·7 i.u./hr, severely depleted of Na⁺; 3, Tr. 4, ACTH 5·3 i.u./hr, moderately depleted of Na⁺. Conventions as in Fig. 1.

No progressive increase in the rate of corticosteroid secretion took place during consecutive collections of blood samples and the observed variations were random only.

Effects of blood transfusion on corticosteroid secretion of Na^+-depleted sheep

In two experiments on Tr. 1 and Tr. 4 (severely depleted of Na^+), the adrenal venous blood was drained through the polythene cannula for a period of $2\frac{1}{2}$–$3\frac{1}{2}$ hr After collection of the first adrenal venous sample the blood lost by this route was continuously replaced by intravenous infusion of compatible blood from a donor sheep in normal Na^+ balance, while the drainage of adrenal venous blood continued. The effect of the loss of adrenal secretion on the composition of the saliva in these experiments has been reported elsewhere (McDonald *et al.* 1958). The first adrenal venous collection, before the blood transfusion, amounted to 43–160 ml. Relative to the sheep's blood volume this initial blood loss was not great. The injection of blood was accompanied by a definite but transient increase in the rates of secretion of cortisol, corticosterone and aldosterone (Table 2). The highest values were recorded in the first sample after blood transfusion began. It was interesting to note that the highest output of aldosterone in the experiment on Tr. 1 was 0·038 mg/hr, much in excess of the combined effect of Na^+ depletion and ACTH stimulation in previous experiments.

TABLE 2. Effect of blood transfusion on corticosteroid secretion by adrenal transplant. Sheep depleted of Na^+ (approx. 600 m-equiv deficit)

Sheep no.	Adrenal blood flow (ml./min)	Secretion (mg/hr)		
		Cortisol	Corticosterone	Aldosterone
Tr. 1.				
Before blood transfusion	2·29	1·20	0·042	0·024
0–51 min	2·67	2·29	0·153	0·038
51–113 min	2·26	1·68	0·091	0·024
113–204 min	2·09	1·10	0·077	0·022
Tr. 4.				
Before blood transfusion	3·40	0·65	0·024	0·009
0·70 min	2·50	0·84	0·043	0·014
70–149 min	2·30	0·52	0·026	0·013

Relationship between adrenal blood flow and corticosteroid secretion

The adrenal blood flow was measured with every collection of adrenal venous blood. It varied considerably from experiment to experiment, but showed only minor fluctuations during any one experiment, which were not related in any way to the corticosteroid secretion. In general, Na^+ depletion was associated with the lowest adrenal blood flow. It was evident that after the sheep were placed on the regime of self-selection of $NaHCO_3$, the adrenal blood flows were much greater, but again subsequent Na^+ depletion was associated with a lower adrenal blood flow than in the undepleted state. For example, the average adrenal blood flow of Tr. 1 when undepleted and receiving $NaHCO_3$ intermittently by rumen tube was 3·60 ± 0·16 (S.E.) ml./min, whereas during Na^+ depletion it was 2·57 ± 0·06 (S.E.) ml./min. When the sheep was determining its own Na^+ intake by selection, the average adrenal blood flow was 8·8 ml./min, whereas during Na^+ depletion caused by withholding the $NaHCO_3$ supplement it was 7·7 ml./min. In the first group the difference between the Na^+-undepleted and Na^+-depleted states was statistically highly significant ($P < 0{\cdot}001$); but in the second group there were insufficient observations on the Na^+-depleted adrenal blood flow to assess the significance of the observed difference. The higher adrenal blood flows found when the sheep determined their own Na^+ intake may have been related to the better general condition of the animals (see Methods), or perhaps to the greater period of time which had elapsed since transplantation. The intravenous injection of ACTH had no effect on the adrenal blood flow in any experiment.

Corticosteroid secretion by the adrenal gland after transplantation in the anaesthetized sheep

During one transplantation operation the blood which flowed through the transplanted adrenal gland immediately after arterial anastomosis was collected and assayed for corticosteroids. At that time the sheep was anaesthetized with cyclopropane. The outputs were as follows: cortisol, 0·580 mg/hr; corticosterone, 0·051 mg/hr; aldosterone, none detected in 60 ml. of plasma; and 11 β-hydroxy-3:17-androstene dione, 0·028 mg/hr. Some cortisone was also detectable. This result is similar to the finding of Fleming & Farrell (1956), that the capacity of the dog's adrenal gland to secrete corticosteroid is not immediately affected by transplantation to the femoral artery.

DISCUSSION

The first general conclusion to be drawn from the results of the foregoing experiments is that the autotransplanted adrenal gland of the sheep secretes significant amounts of biologically active corticosteroids, and is capable of quantitative and qualitative changes in its secretion in response to appropriate stimuli. In agreement with Bush & Ferguson (1953), who examined the corticosteroid secretion by the adrenal gland of anaesthetized sheep *in situ*, we found that cortisol was the predominant corticosteroid in the adrenal venous effluent. In the blood sample taken from a freshly transplanted adrenal gland while the sheep was under anaesthesia the corticosteroid output was similar to that reported by Bush & Ferguson, including the secretion of 11β-hydroxy-3:17-androstene dione. On the other hand, in the conscious undisturbed Na^+-undepleted sheep the quantity of corticosteroids secreted, other than cortisol, was often insufficient for detection in 15–75 ml. of adrenal venous plasma. Small amounts of corticosterone, cortisone and an unidentified Δ^4-3-keto-steroid were detected on some occasions but we were never able to detect 11 β-hydroxy-3:17-androstene dione, even during exogenous stimulation by ACTH. (A substance similar in behaviour to 11 β-hydroxy-3:17-androstene dione was noticed by Reich (1958) to be a break-down product of cortisol.) The reason why 11 β-hydroxy-3:17-androstene dione was found under conditions of anaesthesia and surgical trauma, but not during exogenous ACTH stimulation, is obscure. Aldosterone was secreted in amounts sufficient for detection in 10–15 ml. of adrenal venous plasma only when the animals were depleted of Na^+, but not when in normal Na^+ balance.

The variation in the rate of unstimulated corticosteroid secretion from experiment to experiment, although marked, fell within a range which was considerably less than the ACTH-stimulated secretion. In any one experiment the control secretion rates measured at the beginning and end were approximately similar, and four to five consecutive bleedings taken at 30 min–1 hr intervals over a period of 2·5–3 hr from Na^+-depleted and undepleted sheep showed only a narrow range of variation in the output of individual steroids in any one experiment. Since the different experiments were separated by intervals of 3 weeks to 6 months, it is more likely that the variation from one experiment to the other is explicable on the basis of periodic alteration in the secretory behaviour of the gland rather than of unrecognized excitement of the animals during the experiments. In support of this it was noted that the effect of the same dose of ACTH on the corticosteroid output was least when the output during the control period was least. Many more experiments, involving a greater number of sheep, would be required to establish definitely the variations in corticosteroid output by the transplanted adrenal gland in the normal state, and the matter is emphasized here merely to indicate the necessity of control samples at the beginning and end of each experiment.

ACTH had an effect on corticosteroid secretion which was quantitatively large, and appeared to affect the secretion of corticosterone relatively more than cortisol. If aldosterone was secreted in detectable amounts as a result of Na^+ depletion its secretion rate was also increased. The maximum outputs of cortisol and corticosterone were similar in the undepleted and Na^+-depleted states, although slightly higher values for cortisol and lower values for corticosterone were found in the former state. The maximum rate of secretion of cortisol (F) was of the order of 3–4 mg/hr, of corticosterone (B) 0·08–0·2 mg/hr, and of aldosterone 0·020–0·025 mg/hr. In the undepleted sheep the cortisol output increased during ACTH stimulation from a mean of 0·29 mg/hr to a mean of 3·6 mg/hr, an increase of approximately 12 times, whereas corticosterone increased from a mean of 0·003 to 0·112 mg/hr, an increase of approximately 37 times. In severe Na+depletion cortisol increased from a mean of 0·74 mg/hr to a maximum of 3·04 mg/hr, an increase of 4 times, and corticosterone from a mean of 0·031 mg/hr to 0·14 mg/hr, an increase of approximately 4–5 times. Thus as a general rule the *F*:*B* ratio fell greatly during ACTH stimulation in the undepleted sheep and only slightly in the depleted state (Table 1).

This feature of ACTH stimulation in the two different states of Na^+ balance illustrates the difficulties of interpretation of experiments in which anaesthesia and surgical trauma have affected the metabolic state of the subject. For example, Bush (1953) found no change in the *F*:*B* ratio of adrenal venous blood during ACTH stimulation in a variety of anaesthetized experimental animals, and Grant, Forrest & Symington (1957) found that the intravenous injection of ACTH in human patients during surgical exposure of one adrenal gland caused an increase in the secretion rate of cortisol, but not of corticosterone, i.e. the *F*:*B* ratio rose. On the other hand, Petersen (1957) found that the intravenous injection of ACTH in the *conscious* human subject caused a sustained increase in both cortisol and corticosterone concentrations of peripheral blood, with a fall in the *F*:*B* ratio. These contradictory findings suggest that in conditions of 'stress', such as anaesthesia, surgical trauma, and Na^+ depletion, the adrenal gland is already in a state of altered activity due to the endogenous secretion of ACTH (Engell, Bro-Rasmussen & Burns, 1958), and further stimulation of the gland has a variable effect on corticosteroid secretion, depending on the degree of endogenous stimulation. Holzbauer & Vogt (1957) noted that the effect of surgical trauma and pentobarbitone anaesthesia on the corticosteroid secretion by the rat's adrenal gland was very variable, and found it necessary to conduct experiments during maximal stimulation of the gland by ACTH injection.

Since the Na^+-depleted sheep must have had a smaller blood volume, and probably cardiac output, than the Na^+-undepleted sheep, the concentration of injected ACTH in the circulating blood of the former must have been considerably higher than in the latter, yet there was no great difference in the maximum corticosteroid secretion rates. Thus it is likely that the dose of 5–6 i.u./hr given intravenously was sufficient for maximal stimulation of the transplanted adrenal gland. This rate of ACTH injection would have given a peripheral blood concentration of 0·10–0·06 i.u./l. in the Na^+-undepleted sheep, which is within the range quoted by Macchi & Hechter (1954*a*; 0·1–0·01 i.u./l.) as sufficient for maximal stimulation of the isolated perfused calf adrenal. These authors (1954*b*) also noted that about 18% of the ACTH effect on corticosteroid secretion persisted 126–144 min after exchanging the perfusate to one without ACTH, whereas with the transplanted sheep adrenal no residual action was noted 90–180 min after the period of ACTH stimulation.

The secretion of aldosterone in significant amounts, when the sheep were depleted of Na+, was clearly demonstrated, and it is of interest to note two features in relation to this secretion. First, an increase in aldosterone secretion during ACTH stimulation (Farrell, Rauschkolb & Royce, 1955; Liddle, Cornfield, Casper & Bartter, 1955; Muller, Riondel & Manning, 1956) was unequivocally demonstrated, the effect being much smaller than on the other corticosteroids (an average increase of approximately twice) and dependent on the initial secretion rate. Secondly, the secretion rate of aldosterone was similar in both the moderately and severely Na^+-depleted states. Thus it seemed that some early consequence of Na^+ deficit affected the adrenal gland so as to initiate or greatly to increase the secretion of aldosterone, and this augmented secretion could be further increased to an apparent limit by the injection of exogenous ACTH, but not by further Na^+ depletion. In other words, some factor in addition to increased ACTH secretion was involved in the adrenal response to Na^+ depletion, as is suggested by the numerous studies already reported on man and experimental animals (Singer & Stack-Dunne, 1955; Gaunt, Rienzi & Chart, 1955; Hernando, Crabbe, Ross, Reddy, Renold, Nelson & Thorn, 1957). Further interpretation of the results on these lines can only be speculative, since not enough data are yet obtained. The circumstances which are known to be associated with Na^+ deficit and hence increased secretion of aldosterone in the sheep are these: (1) reduced plasma Na^+, HCO_3 and pH, with increased plasma K^+ (Denton, 1956, 1957)—the fall in plasma Na^+ (7–12 m-equiv/l.) is greatest in the first 48 hr following $NaHCO_3$ withdrawal, after which plasma Na^+ may approach the control value again (McDonald, unpublished); (2) reduced blood pressure and blood volume (Denton, 1957); (3) increased haematocrit and plasma protein; (4) reduced blood flow through the transplanted adrenal gland; and (5) high dietary K^+ intake.

It will be noted that either one or a combination of the first four factors could readily influence the metabolic status of the adrenal gland. In particular, the reduced blood flow through the gland is of interest since the vascular arrangement in the adrenal tissue, if similar to the cat's adrenal, would be such that a reduction in blood flow could affect the metabolism of the inner zones of the cortex more than the outer zone (Bennett & Kilhan, 1940). Since it is now believed that the outer zones are primarily concerned with aldosterone secretion (Ayres, Gould, Simpson & Tait, 1956), this finding may be of some significance. Although there is some evidence of a diencephalic factor other than ACTH being involved in the regulation of aldosterone secretion (Rauschkolb & Farrell, 1956), the evidence so far presented is not entirely convincing. It is quite possible that there may be a number of non-specific ways of augmenting aldosterone secretion, as is indicated by the blood transfusion experiments, when blood obtained from Na^+-undepleted donor sheep caused a marked increase in aldosterone output by the transplanted gland of Na^+-depleted sheep, in one instance far beyond the effect obtained by the combined stimulus of Na^+ depletion and exogenous ACTH injection. This finding suggests that the effect of blood and tissue extracts on adrenal secretion should be interpreted with a certain degree of caution. Similar non-specific stimulating activity of plasma protein fractions with respect to corticosteroid secretion by incubated adrenal slices have been reported (Roberts, 1957).

Thus it is possible by use of the adrenal transplant preparation to make serial observations on its corticosteroid secretion in conscious undisturbed animals, to repeat experiments on the same animal in different metabolic states, and to correlate the corticosteroid secretion with the manifestations of a change in the metabolic state. The findings indicate that a close study of the behaviour of the adrenal transplant in relation to general and local disturbances of its fluid environment will lead to a clearer understanding of the factors concerned in the regulation of adrenal secretion.

Further experiments are in progress to examine the relationship between corticosteroid secretion and Na^+ balance more closely, and to investigate the general metabolism of the transplant in different physiological states.

SUMMARY

1. The sheep's autotransplanted adrenal gland secretes significant amounts of biologically active $\Delta^4$3-ketosteroids, and is readily accessible for repeated sampling of adrenal venous blood when the animal is conscious and undisturbed.

2. In the conscious Na^+-undepleted state, the predominant corticosteroid in the adrenal venous effluent is cortisol, which is secreted at rates varying between 0·04 and 0·60 mg/hr. Very small amounts of corticosterone, cortisone, and an unidentified $\Delta^4$3-ketosteroid are detectable.

3. In the conscious Na^+-depleted state, aldosterone is always present in the adrenal venous effluent, and is secreted at rates of 0·006–0·024 mg/hr. In moderate Na^+ depletion (200–400 m-equiv deficit) the secretion rates of cortisol and corticosterone are within the Na^+-undepleted range, but in severe Na^+ depletion (400–600 m-equiv deficit) the secretion rates of cortisol and corticosterone are significantly raised. The range of aldosterone secretion rate is the same in both stages of Na^+ depletion.

4. Intravenous injection of ACTH at a constant rate of 5–6 i.u./hr caused a prompt increase in the rates of secretion of cortisol and corticosterone, maximum values of 3–4 mg/hr and 0·08–0·12 mg/hr being reached in 60–100 min. The rate of increase was faster in Na^+ depletion, but the maxima were somewhat lower than in the Na^+-undepleted state. If aldosterone was present in the adrenal venous effluent as a result of Na^+ depletion, ACTH stimulation caused an increase in its rate of secretion to a maximum of 0·020–0·025 mg/hr in every instance, the amount of increase being dependent on the initial rate of unstimulated secretion.

5. Transfusion of compatible blood from a Na^+-undepleted donor sheep to Na^+-depleted sheep with transplanted adrenal glands caused a marked but transient increase in the rates of secretion of cortisol, corticosterone and aldosterone.

6. In a single experiment anaesthesia was associated with the appearance of 11 β-hydroxy-3:17-androstene dione in the adrenal venous effluent from a freshly transplanted adrenal gland.

7. The adrenal blood flow was not affected by intravenous injection of ACTH, but Na^+ depletion caused a highly significant reduction in adrenal blood flow.

8. It is concluded that the autotransplanted adrenal gland is a suitable preparation for the detailed study of adrenal function in the sheep, and the possible significance of some of the findings is discussed.

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EFFECT OF ADRENALECTOMY ON TISSUE HISTAMINE IN RATS

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Changes in tissue histamine can be brought about in rats by adrenalectomy and by histamine liberators such as compound 48/80. Adrenalectomy increases and 48/80 decreases the tissue histamine, but these changes do not occur in all tissues of the rat. Moreover, different tissues are affected by adrenalectomy and by 48/80; adrenalectomy increases the histamine in the wall of the intestine, in the liver and lung, but not in the kidney, spleen and blood (Rose & Browne, 1940), whereas injections of 48/80 greatly reduce the histamine in the skin, skeletal muscle and heart but not in the wall of the gastro-intestinal tract or liver (Feldberg & Talesnik, 1953).

The histamine which can be released by 48/80 is derived from mast cells and it appears that the histamine which is resistant to 48/80 is found in those tissues which contain histaminase. Riley & West (1953) and Riley (1953) found that 48/80 disintegrates mast cells and that there is a close parallel between mast cell count and histamine content in those tissues of the rat from which histamine can be liberated by 48/80. The wall of the gastro-intestinal tract and liver were found to contain few mast cells (Mota, Beraldo, Ferri & Junqueira, 1956) although these tissues contain relatively large amounts of histamine; but this histamine is resistant to 48/80. These tissues on the other hand are rich in histaminase (Schayer, 1953; Haeger & Kahlson, 1952; Waton, 1956). Since adrenalectomy causes not only an increase in histamine in these tissues but also a reduction in histaminase (Karady, Rose & Browne, 1940) the question arose whether the increase in tissue histamine which results from adrenalectomy could be fully accounted for by the reduction in histaminase activity.

The present experiments were performed to find out if adrenalectomy only increases the histamine content of tissues which are rich in histaminase, like the wall of the intestine, or whether it affects also the histamine content of a tissue, like the skin, which contains no histaminase (Waton, 1956). The effect of adrenalectomy was therefore measured on the histamine content of intestinal wall and skin. The problem of whether adrenal cortical hormones affect only the histamine which is resistant to 48/80 was examined by two further groups of experiments. The effect of adrenalectomy was examined on the restoration of histamine in skin depleted of its histamine by 48/80 and on the output of histamine which occurs from perfused limbs spontaneously and in response to 48/80.

METHODS

Two strains of male Wistar rats were used. One (*A*) came from Messrs Tuck and Sons, Raleigh, Essex, and the other (*B*) from the Chester Beatty Institute, London. M.R.C. diet 41 was fed throughout the experiments.

Depletion of skin histamine was effected by intraperitoneal injections of 48/80, twice daily for five days. Two injections of 100 μg/100 g body weight were made on the first day; thereafter the total daily dose was raised by 200 μg/100 g body weight each day, as described by Feldberg & Talesnik (1953). The concentration of 48/80 used was 1 mg/ml.

Adrenalectomy was carried out under ether anaesthesia. Thereafter the rats received 0·4 NaCl (w/v) in their drinking water, unless otherwise stated. In some rats, *demedullation* of the adrenals was performed instead of adrenalectomy. This was done as described by Evans (1936) under ether anaesthesia. These animals received 0·4% NaCl in their drinking water for the first four post-operative weeks and were used for experiment at six weeks. Some of the rats were used for determination of skin histamine and others for measuring the regeneration of adrenal cortical tissue. For this purpose the demedullated glands were dissected free from fat and were weighed on a torsion balance; the absence of chromaffin tissue was checked histologically.

Hind-limb perfusions. Rats of strain *B* weighing between 250 and 370 g were used. The aorta was cannulated under pentobarbitone anaesthesia in order to perfuse the hind limbs with oxygenated Tyrode solution at 37° C at a pressure of 28 mm Hg. All visible branches of the aorta distal to the point of cannulation, except the iliacs, were tied off. The outflow was collected through a polythene cannula in the inferior vena cava; again all visible side branches were tied off. The perfusion rates varied from 60 to 90 ml./hr. Once the outflow had become nearly free from blood it was collected in 15 min samples, which were assayed for histamine. For studies of the effect of 48/80 on the release of histamine, 2 μg 48/80 was slowly injected in a volume of 0·2 ml. into the aorta, care being taken not to raise the perfusion pressure.

Extraction of tissue histamine. The method used was that described by Feldberg & Talesnik (1953). The skin area used for extraction was from the ventral surface of the trunk and consisted of skin covering the lower part of the thorax and the upper part of the abdomen. The intestine used was the upper ileum, the lumen of which had been gently irrigated with Tyrode fluid before it was dried between filter paper and weighed. Acid extracts were stored in the refrigerator and were adjusted to pH 7·0–7·5 just before assay.

Assays of histamine were performed on the arterial blood pressure of cats which were anaesthetized with chloralose (1% (w/v) in NaCl solution 0·9% (w/v), 8 ml./kg) and given atropine sulphate 1 mg/kg intravenously, or on a preparation of guinea-pig ileum suspended in oxygenated Tyrode fluid at 32° C. Two-by-two assay designs were used throughout. All responses to tissue extracts were shown to be abolished by mepyramine maleate 1 mg/kg or 0·1 μg/20 ml. respectively. All values for histamine refer to the base.

RESULTS

The concentrations of histamine in the skin and wall of the small intestine in normal, adrenalectomized and adrenal-demedullated rats

The results are summarized in Table 1 and show that adrenalectomy increases the histamine in the skin but not as much as it increases the histamine in the intestinal wall. This increase in skin histamine was found both in rats of strain *A* and in those of strain *B*, although the normal skin histamine concentration differed greatly in these two strains. Adrenalectomy increased the skin histamine concentration of strain *A* by 27 % and that of strain *B* by 38 %. These increases are small in comparison with the increase of 134 % produced by adrenalectomy in the histamine concentration of the intestinal wall in strain *A*. Demedullation had the opposite effect on the histamine concentration in skin and decreased it by 35 %. These experiments were performed in older rats of strain *A*, but, as is shown by the skin histamine values obtained from unoperated rats of this strain, age had not significantly affected the concentration of histamine in the skin. The demedullated glands taken from six of these animals weighed 10·6 ± 0·8 mg/100 g body weight, whereas those taken from six unoperated rats of the same strain and weight group, similarly caged, housed and fed, weighed 18·4 ± 1·4 mg/kg body weight.

TABLE 1. Concentrations of histamine in skin and small intestines of normal, adrenalectomized and adrenal-demedullated rats

Rats			Concentration of histamine; mean ± S.E. (μ/g tissue)
Strain	Procedures	Range of body weight (g)	
			Skin
A	Unoperated	130–180	30·3 ± 2·17 (29)
A	7–13 days after adrenalectomy	130–200	38·0 ± 3·36 (19)*
B	Unoperated	275–360	18·8 ± 1·83 (6)
B	6–8 days after adrenalectomy	225–370	25·9 ± 1·51 (6)*
A	Unoperated	225–300	27·7 ± 2·26 (12)
A	Six weeks after demedullation	245–290	18·2 ± 2·21 (6)*
			Intestinal wall
A	Unoperated	130–180	16·2 ± 1·74 (10)
A	13 days after adrenalectomy	130–180	37·8 ± 3·24 (8)**

Mean values ± S.E. followed by number of animals in brackets. Significance of differences between the histamine concentrations found in unoperated and operated animals was determined by t test; * $P < 0{\cdot}05$, ** $P < 0{\cdot}001$.

The effect of adrenalectomy on the restoration of histamine which had been depleted by 48/80

The results in Table 2 confirm the previous findings of Feldberg & Talesnik (1953) that by repeated intraperitoneal injections of 48/80 the skin histamine is reduced by 80–90 %, whereas the histamine content of the intestinal wall is not reduced. These results were obtained after 5 days administration of 48/80, and the rats were killed on the sixth day. The Table further shows that the treatment with 48/80 increased the weight of the adrenals by 50 %.

Of sixty-two rats of the strain *A* which had been treated with 48/80 for

Table 2. Effects of 48/80 on histamine in the skin and the walls of the small intestine and on adrenal weight in rats of strain *A* weighing 130–180 g.

Histamine (μg/g tissue)	Untreated	Treated with 48/80
Skin	30·3±2·17 (29)	4·12±0·27 (5)*
Intestinal wall	16·2±1·74 (10)	18·4±2·38 (10)
Adrenal weight (mg/100 g body wt.)	20·1±0·43 (16)	30·9±0·91 (16)*

Mean values ± s.e. followed by the number of animals in brackets. Significance of difference between the means for treated and untreated rats was determined by *t* test; *$P < 0{\cdot}01$.

Table 3. The restoration of skin histamine in rats after treatment with 48/80 without and with subsequent adrenalectomy carried out 1 day after cessation of treatment with 48/80. Males, strain *A*, body weight 130–180

	Histamine in skin (μg/g tissue)		
		Adrenalectomized rats	
Days after cessation of 48/80	Control rats	Saline to drink	Water to drink
5	8·4±0·50 (8)	14·0±2·12 (8)*	11·4±2·17 (5)
8	10·1±0·85 (7)	15·7±0·72 (9)*	15·8±2·40 (4)
14	16·1±1·11 (7)	22·5±1·36 (7)*	30·4±4·37 (4)**

Means ± s.e. followed by the numbers of animals used, in brackets. The significance of difference between the means given by control and operated animals was examined by *t* test; * $P < 0{\cdot}05$, ** $P < 0{\cdot}001$.

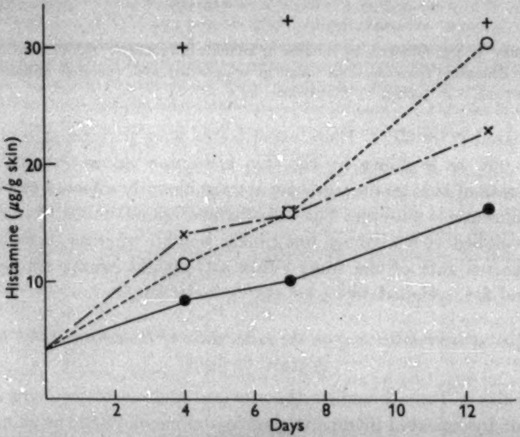

Fig. 1. Restoration of skin histamine after depletion by 48/80 in non-adrenalectomized and adrenalectomized rats (same animals as in Table 3). Ordinates, mean values for skin histamine, abscissae time in days from the completion of treatment with 48/80; adrenalectomy on day 0. Control rats ●—●; adrenalectomized rats maintained on saline ×—×; rats given no saline ○—○; mean values obtained from control rats which received no 48/80 +.

5 successive days, forty were adrenalectomized on the sixth day. Groups of unoperated and of operated rats were then killed after 4, 7 and 13 days for estimation of their skin histamine. The results, which are summarized in Table 3 and Fig. 1, show that the restoration of skin histamine proceeds more quickly in adrenalectomized than in unoperated rats. One group of adrenalectomized rats had been given saline to drink throughout their survival period. The adrenalectomized animals of the other group drank only water post-operatively. It will be seen that the skin histamine of the adrenalectomized rats which drank water had returned to the original level in 13 days, whereas that of the unoperated rats had, at this time, restored only 50% of their skin histamine. In the operated rats which had been given saline to drink throughout the skin histamine had risen more (i.e. to 66%) than in the unoperated animals, but not as much as in the operated animals which had drunk water. There was no significant difference in the skin histamine on the fourth and seventh days after adrenalectomy between operated rats which had received saline or water to drink, but in both groups the skin histamine had been restored to a higher level than in the unoperated animals.

The release of histamine, spontaneously and in response to 48/80, *from the perfused hind limbs of normal and adrenalectomized rats of strain B*

In twelve unoperated rats the spontaneous output of histamine from the perfused hind limbs was 1·24 ± 0·03 μg/100 g body weight/20 min, whereas the output in twelve rats which had been adrenalectomized 6–10 days previously was 0·72 ± 0·01 μg/100 g body weight/20 min. There was therefore a significant reduction in the rate at which histamine was released spontaneously from the perfused limbs of adrenalectomized animals (t calc. = 2·13, $n = 22$, $P = 0{\cdot}05$). However, there was no significant difference in the amount of histamine liberated by 2 μg 48/80 in these two groups of animals. The 48/80 caused the release of 10·8 ± 2·44 μg of histamine from the hind limbs of seven control rats and of 14·3 ± 2·10 μg from those of seven rats of the adrenalectomized group. A second injection of 2 μg 48/80 given about 1 hr later released 6·3 ± 1·9 μg histamine from the limbs of the control animals and 6·6 ± 0·7 μg histamine from the limbs of the adrenalectomized rats.

DISCUSSION

The present experiments show that adrenalectomy increases the histamine not only in a tissue like the wall of the intestinal tract which is rich in histaminase but also in a tissue like the skin which does not contain this enzyme. Although the increase in the skin is less pronounced than in the intestinal wall, reduction in histaminase activity cannot fully explain the rise in tissue histamine observed after adrenalectomy. Reduction in histaminase activity may, however, play a part in the great increase of histamine which occurs in the intestinal wall.

The rise in skin histamine which occurs after adrenalectomy, and which cannot be explained by reduction in histaminase activity, can also not result from an augmented uptake of free circulating histamine in blood, since adrenalectomy does not increase the histamine in blood (Rose & Browne, 1940). Moreover, it is unlikely that the skin histamine is derived from circulating histamine since skin is unable to bind labelled histamine injected into the circulation (Schayer, 1952; Schayer & Smiley, 1954). The present view about the origin of skin histamine assumes that it is derived from histidine (Schayer, 1955, 1956), but there is no histidine decarboxylase in rat skin (Waton, 1956), so that the mechanism by which histamine is stored in rat skin is unknown.

The finding that the spontaneous output of histamine from perfused hind limbs is less in adrenalectomized rats than it is in normal rats suggests that the histamine in skin and muscle is more firmly fixed after adrenalectomy. This finding is of interest in connexion with the observations of Schayer (1955, 1956) that the skin of adrenalectomized rats had a greater ability to bind histamine formed *in vitro* from histidine than the skin of normal rats. Thus the increase in skin histamine after adrenalectomy may result from an increase in the firmness with which histamine is bound in the tissues in the absence of cortical hormone. This explanation may also account for the finding that, in skin depleted of its histamine by 48/80, histamine restoration proceeds more rapidly after adrenalectomy. This effect of adrenalectomy may be associated with the changes in mineral metabolism which occur in the absence of cortical hormones. Rose & Browne (1940), for instance, found a greater increase in the histamine of the intestinal wall after adrenalectomy in rats which drank water than in those which drank saline, and the present experiments show that the restoration of skin histamine in adrenalectomized rats was greater when they drank water instead of saline during the survival period.

SUMMARY

1. Adrenalectomy increases the mean histamine concentration of skin by approximately 30% but that of the intestinal wall by 134%.

2. Demedullation of the adrenals reduces the histamine concentration of skin by 35%.

3. Restoration of skin histamine, reduced by 48/80, is quicker if the rats are subsequently adrenalectomized, particularly when they are not treated with sodium chloride. Restoration of skin histamine is then complete at 13 days, whereas in normal rats restoration in this period only reaches the 50% level.

4. Adrenalectomy reduces the spontaneous release of histamine from perfused hind limbs but not the release produced by 48/80.

The greater part of this work was done during the tenure by A. L. Bartlet of a D.S.I.R. research studentship.

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INHIBITION AND EXCITATION OF RETICULO-RUMEN CONTRACTIONS FOLLOWING THE INTRODUCTION OF ACIDS INTO THE RUMEN AND ABOMASUM

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Microbial fermentation of ingested carbohydrate leads to the production of large amounts of organic acids in the forestomach of ruminants. The chief end products are the short-chain fatty acids, namely acetic, propionic and butyric, and their total concentration in rumen liquor varies from approximately 50 to 160 mM depending upon the time of sampling in relation to feeding. The pH of the rumen digesta of sheep varies from 5·4 to 7·3 (Phillipson, 1942). Some of the possible mechanisms which may operate in keeping the pH within this range were listed by Scarisbrick (1954).

There have been many reports of rumen digesta pH values below 5·0 and as low as 4·2; these acid conditions were associated with high concentrations of lactic and fatty acids (Hungate, Dougherty, Bryant & Cello, 1952; Scarisbrick, 1954; Briggs, Hogan & Reid, 1957). Frequently an inhibition of the rumen contractions accompanied the low rumen pH values. Clark & Lombard (1951) found that the rumen contractions of sheep were depressed in amplitude or completely inhibited by the introduction of alkali into the rumen; similar responses were obtained when alkali was injected intravenously. The conclusion drawn by Clark & Lombard from their experiments was that the inhibition of rumen contractions by alkali was the result of changes in the acid–base balance of the blood. Phillipson (1955) considered this conclusion was unjustified and suggested the possibility of inhibition from stimulation of sensory nerve endings in the rumen epithelium.

The investigations reported in this paper were an attempt first to discover the relationship between the pH of the rumen contents and the motility of the forestomach, and secondly to find out whether any effects produced were caused by peripheral stimulation or were the result of changes in the blood acid–base balance consequent upon absorption. A preliminary account of this work has been published (Ash, 1956).

METHODS

Surgical preparations. Two ebonite cannulae, 2 cm in diameter, were fitted into the dorsal rumen sacs of Blackface and Shetland sheep. Large cannulae, diameter 9 cm, were inserted into the dorsal rumen sacs of Greyface sheep weighing at least 50 kg (Ash, 1957). Some sheep, previously fitted with rumen cannulae, underwent a second operation in which an ebonite cannula 1·3 cm in diameter was inserted into the body of the abomasum. The carotid artery was exteriorized in a loop of skin on the right side of the neck. Bilateral splanchnic nerve section together with removal of the first three lumbar ganglia, adrenal and renal plexuses and the majority of the coeliac ganglia, was carried out on two sheep. Silver electrodes shielded with polythene were applied to the dorsal and ventral vagus nerves in the thorax of one sheep with the large lumen cannula (Dziuk & Sellers, 1955). The operations were performed aseptically and under pentobarbitone anaesthesia. No experiments were carried out on any of the animals until 4–6 weeks after the cannula operations or 7–14 days after sympathectomy or application of the vagal electrodes. All the sheep were trained to stand quietly and for long periods in a recording stand.

Recording. The contractions of the reticulum and in some experiments those of the dorsal rumen sac were recorded. Balloons inflated with air were placed in the reticulum and dorsal rumen sac through the anterior cannula of double cannulated sheep; the posterior cannula was used for introducing solutions into the rumen. The balloons were connected to water manometers which recorded the contractions on a kymograph. The volume of the balloons varied in different experiments from 30 to 100 ml. at +9 to +15 cm water pressure. In each experiment the basal volume and pressure in the recording system were kept constant.

pH measurements. The pH of all the solutions and of the samples taken from the rumen was measured with a glass electrode and a Cambridge or Pye meter. Samples of buffered solutions were siphoned from the rumen into warm beakers and the pH measured immediately at 39° C.

Carotid blood pH was measured by the method of Nims & Marshall (1938) and a continuous record was obtained using the recording method described by Dobson (1957). In conscious animals the skin of the carotid loop was infiltrated with 0·5 % (w/v) lignocaine hydrochloride and incised along its length, and the artery was exposed and cannulated. Connexions between the arterial cannulae and the glass electrode chamber were made with polythene tubing. The glass cannulae and the chamber containing the glass electrode, saturated KCl bridge and thermometer were exposed to dimethyl-dichloro-silane (British Drug Houses Ltd) vapour before being used. Blood clotting was prevented by injecting 14–20 mg of heparin (Roche Products Ltd) /kg body weight intravenously; a further dose of 4–6 mg/kg was injected after 3 hr when the recording period lasted up to 7 hr.

The glass electrodes were calibrated at the working temperature of the solutions and circulating carotid blood with 0·05 M potassium hydrogen phthalate and 0·05 M sodium borate (Hopkin and Williams Ltd, Analytical Reagents).

Analytical. The concentration of CO_2 in the samples of arterial plasma was measured by the method of Van Slyke & Neill (1924). Samples were drawn from a side arm of the electrode chamber over mercury into small tubes which were sealed and centrifuged. The concentration of total steam-volatile fatty acid in whole blood was estimated by steam distillation (Kiddle, Marshall & Phillipson, 1951) and that of lactate by the method of Barker & Summerson (1941). With few exceptions duplicate analyses were performed. The total volume of blood necessary to provide samples for all analyses did not exceed 12 ml.

Nerve stimulation. A square wave electronic stimulator was used for stimulation of the vagus nerves.

General. Uniform mixing of solids or liquids with the normal rumen contents is unlikely to take place immediately after their introduction. In these experiments digesta were removed from the forestomach through a cannula, the sacs were washed clean with tap water or sodium chloride solution 0·9 % (w/v) at 39° C and then solutions of known composition were introduced.

The expression 'forestomach' is used in this paper to refer only to the rumen and reticulum, in order to avoid cumbersome reference to these two organs. In its strict sense it refers to the rumen, reticulum and omasum combined and is derived from the German 'Vormagen'.

RESULTS

The effect of emptying the forestomach on its contractions

It was necessary to starve the sheep 18–24 hr before emptying the digesta from the forestomach; during this time the consistency of the digesta became thin and this facilitated their removal. After fasting and before emptying, the reticulum contractions were of the characteristic biphasic type. It was found that not only did the time interval between contractions vary from 36 to 200 sec in different experiments, but that the frequency showed some variation throughout the course of each experiment. Periods of rumination were observed and on some occasions these were caused by the manipulations necessary to insert the recording balloon into the reticulum. The dorsal rumen sac contracted once or twice after each reticulum contraction. The pH range of the forestomach digesta after fasting was 6·5–7·0; the volume of the digesta removed varied from 1·8 to 4·0 l.

Emptying and washing the forestomach had no consistent effect on the pattern of the reticulum contractions. Three main variations occurred, namely: (i) the frequency of the reticulum contractions was the same before and after emptying; (ii) the frequency was increased after emptying and gradually decreased until it was equal to or less than that of the pre-emptying period; (iii) there was complete absence of reticulum contractions. The third type of response was rare, and when the animal was induced to feed reticulum contractions appeared immediately, only to disappear when feeding stopped. When this response to emptying was obtained the experiment was discontinued.

The form of the reticulum contractions after emptying was biphasic and it was not uncommon for a sheep with an empty forestomach to attempt to ruminate.

Contractions of the dorsal rumen sac were either small in amplitude or absent, as indicated by changes in pressure in the recording balloon. This was an artifact due to a relatively small recording balloon in a large empty sac, for the walls of the rumen did not collapse after its contents were removed.

The presence of the recording balloon in the reticulum may have modified the pattern of its contractions but the contractions were not initiated by the balloon. Cycles of contractions were observed on a fluoroscopic screen after emptying the forestomach and rinsing with a 20% (w/v) suspension of barium sulphate. Furthermore, movements of the forestomach have been observed directly through a large-diameter fistula after removal of the digesta (Ash, Benzie & Phillipson, 1957).

Motility continued in the empty forestomach for at least 9 hr.

The effect of rumen liquor, neutral and alkaline solutions

Nine experiments were performed in which buffered solutions at pH 7·9–10·1 were introduced into the empty rumen. The total concentration of the solutions was 0·1 M. It was found that sodium phosphate (pH 7·9–8·1) and glycine -NaOH -NaCl solutions (pH 9·2–10·1) caused no greater excitatory or inhibitory effects on the reticulum than rumen liquor and neutral solutions; any effects were usually transient.

In one experiment 3 l. of rumen liquor were adjusted to approximately pH 9·0 with 0·5 M-Na_2CO_3. When this solution was introduced into the rumen, it caused no immediate inhibitory effect but after 10 min the sheep ruminated for a 5 min period. After 30 min there was a small decrease in amplitude of the reticulum contractions and the interval between each cycle was 70–95 sec compared to 60–80 sec in the control period.

During the time the alkaline solutions were in the rumen their pH decreased slowly, e.g. a 0·1 M buffered solution of sodium phosphate decreased from an initial value of pH 8·0 to pH 7·5 in 86 min. The mixture of rumen liquor and Na_2CO_3 decreased from pH 9·1 to pH 8·0 in 95 min. It was concluded from these experiments that there was no direct effect of an alkaline pH of the forestomach contents on the contractions of the reticulum and rumen.

The effects of fatty acid solutions

Buffered solutions containing 200 mM acetate at pH 5·0 inhibited the contractions of the reticulum. In one experiment the contractions were inhibited completely for 22 min, when they suddenly reappeared and gradually increased in frequency to reach the control value. The pH of the solution when recovery began was 5·5. The effect of an acetate solution at pH 5·0 is shown in Fig. 1. In this experiment complete inhibition was not produced but the frequency decreased considerably over a 15 min period. In other experiments the amplitude of the contractions decreased and their frequency increased; this effect usually lasted for about 5 min.

Further additions of acetic acid to the solution in the rumen reduced the pH to 4·6–4·8 and again the amplitude and the frequency of the contractions were reduced. Similar responses were produced by 100–200 mM acetate solutions at pH 4·4–4·5 although a decrease in frequency tended to be the predominant response at these pH values.

At pH 3·6–4·0 acetate solutions (100–200 mM) always caused marked inhibitory effects. Complete inhibition of the reticulum contractions was the usual response but in some experiments this was delayed for 60–180 sec after introducing the solution into the forestomach. When inhibition was delayed the reticulum contractions which followed the introduction of the solution diminished progressively in amplitude. Not infrequently these contractions were increased in frequency. Complete inhibition lasted 30–90 min and the pH values at which motility returned were usually below 5·0. Recovery was signalled by the appearance of small biphasic contractions, the frequency of which was slightly less than or equal to that of the control period. Buffered solutions of sodium propionate or sodium butyrate had similar effects.

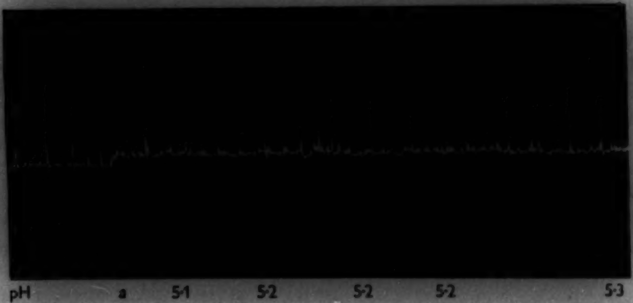

Fig. 1. Inhibition of reticulum contractions by an acetate buffer in a conscious sheep. At the signal *a*, 2·5 l. of a 200 mM acetate buffer at pH 5·0 were introduced into the forestomach. From above downwards: reticulum contractions; signal; time marker 30 sec; pH values of samples of buffer taken from the rumen at intervals marked by the signal.

All fatty acid solutions caused systemic effects and these were most marked following the introduction of buffered solutions of butyrate. The chief symptoms were some apparent discomfort and an increase in the respiration rate; after 10–15 min the sheep became dull. These symptoms rapidly disappeared after the solutions were drained from the forestomach.

The effect of hydroxy-, tricarboxylic and mineral acids

Solutions containing 100–200 mM lactate at pH 3·6–4·0 never caused any appreciable inhibitory effects. Some decrease in the frequency of the reticulum contractions was observed in one experiment, but in five experiments the frequency showed a small but persistent increase. Rumen liquor adjusted to pH 3·7 with lactic acid did not inhibit contractions; the concentration of lactic acid in the mixture was 37 mM. Although the concentration of fatty acid in the liquor was not estimated it was most probably very low, since it was obtained from a sheep that had fasted for 24 hr.

A 200 mM lactic acid solution at pH 2·5 caused an immediate and complete inhibition of the reticulum contractions in one experiment; the contractions reappeared 80 min later when the pH value of the solution in the rumen was 3·6. In two further experiments the amplitude of the contractions decreased progressively with some decrease in frequency, but inhibition was not complete. Complete inhibition was observed in a further experiment 14 min after introducing a 200 mM lactate buffer at pH 2·5.

Similar experiments with buffered solutions of 167 mM-HCl–sodium phosphate at pH 4·0, 167 mM-HCl–glycine at pH 2·9, and 167 mM citric acid–disodium hydrogen phosphate at pH 3·7–4·1 did not cause any appreciable inhibition. It is remarkable that the pH of the rumen contents can be reduced to these extremely low values without affecting motility or causing any symptoms. Clearly, the inhibitory effects observed with acid solutions were not directly dependent on the pH value of the solution.

The dependence of inhibition on the presence of fatty acid at pH 4–5

Since inhibition was produced in some experiments at pH 2·5 with lactate, it cannot be stated that inhibition was a specific effect of fatty acid. However, inhibition occurred when simple fatty acid buffers at pH 4–5 were introduced, whereas non-fatty acid buffers did not cause inhibition. Buffered solutions of lactate containing only 50 mM butyrate inhibited contractions. Generally, the responses with 50 mM butyrate were more marked than those with 50 mM acetate, which on occasions failed to inhibit. All the responses with 50 mM fatty acid combined with lactate buffers were smaller than those with 100–200 mM fatty acid buffers.

The effect of vagal stimulation during acid inhibition

Danielli, Hitchcock, Marshall & Phillipson (1945) found that fatty acids were rapidly absorbed from the forestomach and the rate of penetration increased when the pH value of the solution was lowered. The experiments of Dobson & Phillipson (1956) indicated that the penetration of both lactic and mineral acids through forestomach epithelium was slow, even at pH 4·0. The mechanism of fatty acid inhibition at pH 4–5 appeared to be related to the rapid penetration, and the possibility of inhibition being due to penetration of fatty acid into the musculature of the forestomach and causing a neuromuscular block was investigated.

Electrical stimulation of the peripheral end of the cut left cervical vagus, after the introduction of a 200 mM acetate buffer at pH 3·6 into the forestomach of an anaesthetized sheep, caused contraction of the reticulum and rumen. The contractions of the reticulum were monophasic. In a conscious sheep stimulation of the ventral vagus nerve during acid inhibition also caused monophasic contractions of the reticulum (Fig. 2). There was no significant difference before and after acid inhibition in the force of the reticulum contractions to a constant voltage and frequency of vagal stimulation. These results indicated that acid inhibition was not caused by a neuromuscular blocking mechanism.

The effect of acid buffers after partial sympathectomy

Whilst it was unlikely that complete sympathetic denervation of the abdominal viscera was achieved, it can be stated with certainty that the splanchnic nerves and the main fibres and ganglia forming the renal, adrenal and coeliac plexuses were divided. Fatty acid buffers still caused inhibition in the two sheep on which this operation had been performed; the onset and duration of the inhibitory effect were similar to those observed before sympathectomy.

Fig. 2. Contractions of the reticulum in response to stimulation of the ventral vagus nerve during acid inhibition. At the signal *a* 3 l. of 100 mM lactate + 50 mM butyrate buffer at pH 4·2 were introduced into the forestomach; the buffer was drained at *b*. At each signal *s*, the vagus nerve was stimulated (10 V, 50 c/s, 0·5 msec) for 15 sec. From above down: reticulum contractions; signal; time marker 30 sec.

The effect of acid buffers on the pH, concentration of total CO_2 and fatty acid in the carotid blood

Fatty acid buffers at pH 4·0 caused a marked decrease in the pH value of the carotid blood of anaesthetized sheep. This depression began within 60 sec of introducing the solution into the forestomach and continued to decrease until the pH value was as much as 0·4 of a pH unit below its control value. When these solutions were drained from the forestomach, the pH value of the carotid blood rapidly recovered, but not necessarily to its previous value. Lactate buffers at a similar pH and concentration did not have this effect on the pH value of the carotid blood (Fig. 3).

A further series of experiments was carried out in which the pH of the carotid blood and reticulum contractions were recorded simultaneously in conscious sheep. Figure 4 shows the effects of an acetate buffer. Inhibition began before any appreciable fall in the carotid blood pH value and contractions frequently returned before recovery of the blood pH, and while the concentration of fatty acid was still high (Fig. 5). The concentration of steam-volatile fatty acid in the carotid blood increased rapidly to values as high as 15–18 mM.

Experiments with lactate, glycine–HCl and citric acid–sodium phosphate buffers showed that these did not cause rapid and precipitous decreases in the blood pH value. However, when these solutions were introduced serially into the forestomach of a sheep and the forestomach was drained and rinsed after each solution, the carotid blood pH value and the concentration of total CO_2 decreased progressively. At the start of the experiment the blood pH value was 7·45 and the concentration of CO_2 22·0 mM; at the end of the experiment these values were 7·33 and 15·9 mM, respectively. There was no inhibition of reticulum contractions with these solutions and at the end of the experiment the contractions were of regular frequency and good amplitude. There was no increase in the concentration of lactate in the carotid blood with the lactate buffer.

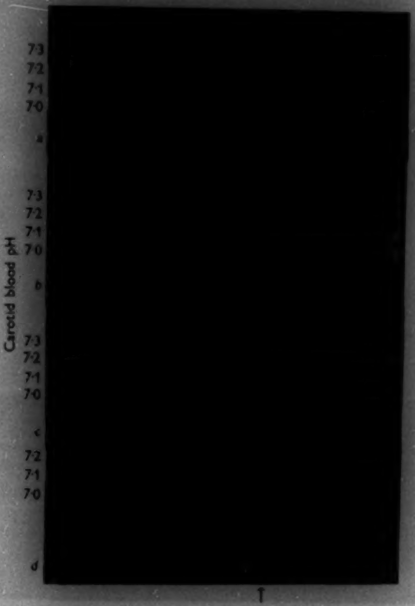

Fig. 3. The effect on carotid blood pH of *a*, acetate; *b*, propionate; *c*, lactate and *d*, butyrate buffers. In each record 3 l. of 200 mM buffer at pH 4·0 was introduced into the forestomach at the first signal; the buffer was drained at the end of each panel or at the arrow in panel *d*. Approximately a 10–15 min break between records *a*, *b*, *c*, and between the arrow and the next part of the record in *d*. From above down in each panel; carotid blood pH with calibration; signal; time marker 30 sec. Sheep anaesthetized with pentobarbitone.

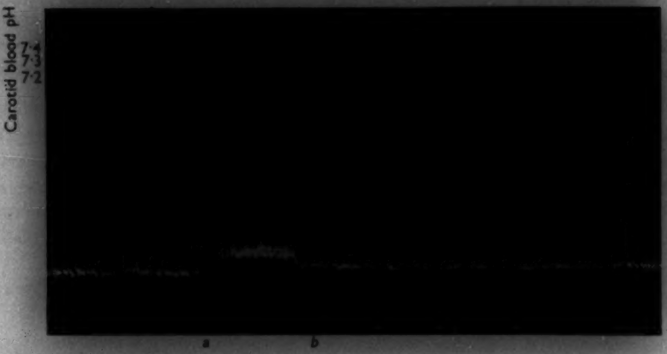

Fig. 4. The effect of an acetate buffer on the pH value of the carotid blood and on reticulum contractions. At the signal *a*, 3 l. of a 200 mM acetate buffer at pH 4·0 was introduced into the forestomach; the buffer was drained at *b*. From above down: carotid blood pH with calibration; reticulum contractions; signal; time marker 1 min. Conscious sheep.

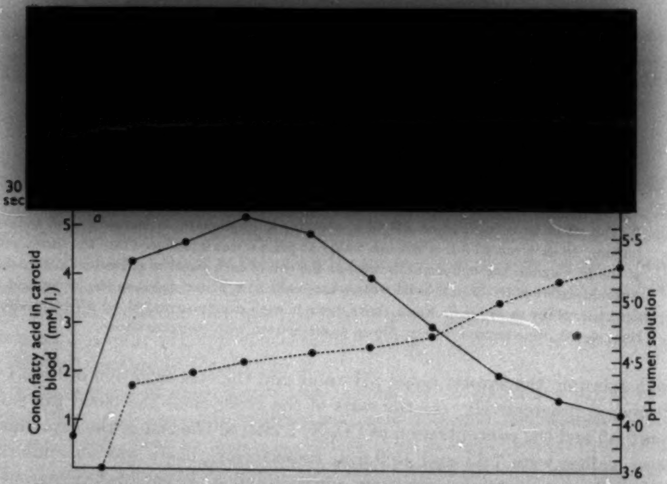

Fig. 5. The effect of an acetate buffer on the concentration of fatty acid in the carotid blood and on the reticulum contractions. At signal *a*, 3 l. of a 200 mM acetate buffer at pH 3·6 was introduced into the forestomach. From above down: reticulum contractions; signal; time marker 30 sec; ●—●, concentration of fatty acid in carotid blood; ●···●, pH of buffer in the forestomach. Conscious sheep.

Inhibition caused by fatty acid vapour

Reticulum contractions were inhibited when the interior of the forestomach was acidified with acetic, propionic or butyric acid vapour. A stream of air was bubbled through concentrated acid and directed through a rumen cannula. Under these conditions inhibition was caused without producing any appreciable changes in pH or in concentration of CO_2 and steam-volatile fatty acid in the carotid blood (Fig. 6).

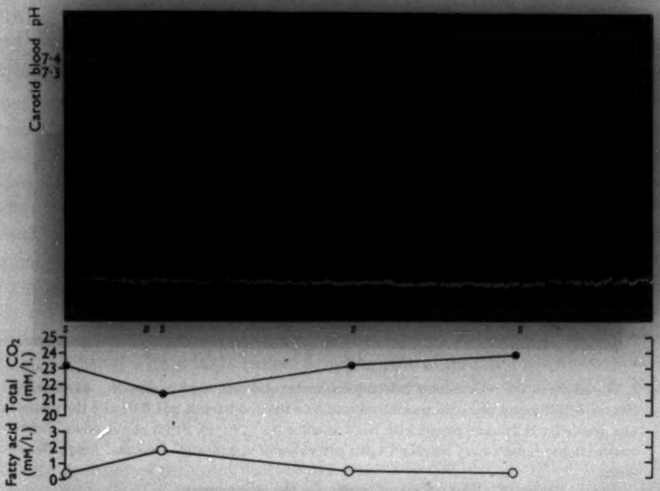

Fig. 6. Inhibition of reticulum contractions by acetic acid vapour. At the signal *a*, acetic acid vapour was blown into the forestomach through a cannula. From above down: carotid blood pH record with calibration; reticulum contractions; signal; time marker 1 min; ●—●, concentration of total CO_2 in carotid plasma; ○—○, concentration of fatty acid in carotid blood. Conscious sheep.

Acidifying the rumen by this method was not very satisfactory because, although no distension was caused, it was possible that some cooling and mechanical stimulation occurred. When a stream of air, CO_2 or oxygen was used there was either no inhibition or inhibition of very short duration. The pH and concentration of acid in the thin layer of fluid on the epithelium were not known, but the concentration of acid was most probably high. The pH value of the residual fluid in the forestomach after introducing fatty acid vapour was 4·5–5·0.

The effect of solutions applied to localized areas of the forestomach

It was possible to apply buffered solutions to localized areas of the forestomach of sheep fitted with a large-diameter rumen cannula. 50 ml. of 100–200 mM acetate or butyrate buffers (pH 3·1–5·0) injected into the cavity of the reticulum, over the anterior pillar of the rumen or into the terminal 10 cm of the oesophagus, did not have any appreciable effect. The responses obtained consisted of an increase or a decrease in the frequency of the reticulum contractions confined to one or, at the most, three cycles. Since responses of a similar nature were obtained on occasions with NaCl solution 0·9% (w/v) it is highly probable that there was some mechanical stimulation of these areas, for they are known to be extremely sensitive to mechanical stimulation (Ash & Kay, 1957).

Fig. 7. The effect on the reticulum of introducing acetic acid into the abomasum. At the signal *a*, 100 ml. of 167 mM acetic acid was introduced into the abomasum, pH 3·0; at *b* the abomasum was drained, pH 2·0, and rinsed with NaCl solution 0·9% (w/v). From above down: reticulum contractions; signal; time marker 1 min; pH value of samples of abomasal fluid. Conscious sheep.

The effect of acids in the abomasum

Titchen (1953, 1958) showed that when the pH value of the abomasal contents of decerebrate preparations was lowered to 1·0 or less with 0·2 N-HCl or H_2SO_4 reticulum contractions were stimulated. Since inhibition of the reticulum did not depend entirely on the pH value of the forestomach contents, experiments were performed in which fatty or lactic acids were introduced into the abomasum and the effect on the reticulum observed. In these experiments both the forestomach and the abomasum were washed clean with NaCl solution 0·9% (w/v) after the digesta had been removed.

An increase in the amplitude or frequency of the reticulum contractions, or both effects, could be produced when 100 ml. of unbuffered 160–200 mM acetic, butyric or lactic acid was introduced into the abomasum. The response usually began within 90–120 sec of introducing the acids. In some experiments the effect continued for 25–50 min, although there was a gradual decrease in amplitude and frequency with time. As the effect declined, the pH value of the abomasal contents decreased. A typical response to acetic acid is illustrated in Fig. 7 where a decrease in the pH of the contents is associated with a gradual decrease in the effect. Usually the pH of the abomasal contents was 2·0–3·0 but never decreased below 1·3. On occasions the interval between successive contractions was only 10–15 sec and many contractions were polyphasic. When the organic acid solutions were drained and the abomasum rinsed with NaCl solution 0·9% (w/v) the effect was abolished.

DISCUSSION

There is no conclusive evidence as to the origin of the regular cycles of reticulo-rumen contractions which occur in the conscious ruminant. Titchen (1953, 1958) considered that contractions of the reticulum of the decerebrated sheep, goat or calf may result from the sum of sensory stimuli from the rumen, omasum, abomasum and the reticulum itself. Conditions in the omasa and abomasa of the conscious animals used in the experiments reported in this paper were unknown; it is possible that the forestomach contractions were reflex in origin. Those preparations in which reticulum contractions were absent after emptying the forestomach were the exception; contractions appeared when those sheep were fed and disappeared when feeding stopped. Emptying the forestomach through a small cannula involved manipulation of the abdominal wall, and the possibility of some form of inhibitory stimulus arising from this procedure cannot be ignored. In thirty-three experiments on six sheep fitted with a large rumen cannula, reticulum and rumen contractions were never absent after emptying the forestomach (R. W. Ash & R. N. B. Kay, unpublished observations). Emptying the forestomach of these sheep was easy and there was the minimum of manipulation of the abdominal wall. A large number of different stimuli are known to modify the pattern of reticulum and rumen contractions reflexly (Phillipson, 1939; Titchen, 1953, 1958; Ash & Kay, 1957). Variability in the pattern of forestomach contractions was therefore to be expected in preparations in which all possible sources of reflex stimulation were not controlled. The important feature of the sheep with an empty forestomach was that the frequency and amplitude of the reticulum contractions maintained a reasonably constant pattern for long periods.

The results of the experiments show that there was no threshold pH value of the forestomach contents for acid inhibition. The pH value at which inhibition occurred depended to a large extent upon the molecular structure and concentration of the acid. That inhibition was dependent upon the molecular structure was shown by the fact that 100–200 mM fatty acid buffers at about pH 4·0 caused inhibition, whereas lactate, citric acid–phosphate and glycine–HCl buffers at a similar pH and concentration did not inhibit. When the effects of 50 and 200 mM acetate solutions at pH 4·0 were compared there was a greater effect with the more concentrated solution.

Since inhibition was caused by fatty acid vapour without appreciably affecting the pH value, total CO_2 and fatty acid concentrations of the carotid blood, it appeared that a peripheral mechanism could be responsible for the inhibition. Spraying the wall with vapour covered a large area of the epithelium and it was likely that a high concentration of fatty acid was produced within a thin film of water.

Failure to produce inhibition by the local application of 100–200 mM solutions at pH 3·1–5·0 may have been due to the area treated being a small one and to the rapid neutralization of the solution. In order that receptors should be stimulated by either acids or alkalis, the test solution must gain access to the nerve endings, so that the local pH is increased or decreased to the threshold of the receptor. Stimulation will therefore depend upon three main factors, namely: (i) the relationship of the receptor to the surface exposed to the solution; (ii) the ability of the solution to penetrate to the receptor when the two are separated; and (iii) the test solution must remain at or above a certain hydrogen ion concentration. Consideration must be given also to the possibility that receptors may be stimulated as a result of local changes in CO_2 tension rather than by the concentration of hydrogen or hydroxyl ions as such.

Many explanations have been put forward to account for the high pH threshold of receptors to fatty acids. Pfaffman (1941) found that acetic acid solutions stimulated receptors in the cat's tongue at a higher pH value than did HCl. Thomas & Crider (1940) found also that acetic acid stimulated the flow of pancreatic secretion at a higher pH value than did HCl when 0·15 M buffered solutions were applied to the intestinal mucosa of dogs. The conclusion of Pfaffman and of Thomas & Crider was that the differences in threshold were due to the buffering power of the solutions rather than to differences in the molecular structure of the acids. Solutions of acids are maximally buffered at their pK values and this value for acetic acid is 4·7 and for propionic and butyric acids 4·8 (Clark, 1925; Britton, 1955). The percentage of free acid in the solution will depend upon the pH of the solution and the pK value of the acid, i.e. in equimolar solutions of lactic and acetic acid at pH 4·0 there is a higher percentage of free acid in the acetate than in the lactate solution; the pK value of lactic acid is 3·8 (Britton, 1955). An examination of the results suggested at first that inhibition was related to the amount of free acid in the buffers. Furthermore, the rate of penetration of free fatty acid through rumen epithelium is high and much higher than that of fatty acid anion (Danielli *et al.* 1945) but this alone does not explain the greater effect of butyric acid when compared with acetic acid. The phenomenon of an increased effect with increasing length of the carbon chain in the fatty acid series is well known in biological systems.

Clearly, the high concentration of fatty acid in the peripheral blood which occurred when fatty acid solutions were introduced into the forestomach cannot be ignored as a possible cause of inhibition, for Le Bars, Lebrument, Nitescu & Simonnet (1954) found that intravenous injections of the sodium salts of acetic, propionic and butyric acids inhibited the forestomach contractions of sheep. However, it was shown in the experiments reported in this paper that high concentrations of fatty acid in the peripheral blood were not an essential for inhibition to occur.

Reflex or central excitation of the sympathetic nerves to the forestomach, or a release of adrenaline, are unlikely to be the mechanisms of inhibition, for the response was not prevented by division of the splanchnic nerves and partial lumbar sympathectomy. The response could be due to reflex inhibition of vagal motor impulses to the forestomach, the afferent fibres of the reflex arc also travelling in the vagus nerves.

A further feature was the compound effect caused by introducing acid buffers. In many experiments fatty acid buffers caused the frequency of the reticulum contractions to increase while the amplitude decreased. Such a response could be caused by the presence of two types of receptors in the rumen wall, both of which are stimulated by acid though having different central effects.

Receptors which respond specifically to alkaline solutions at pH 8 and above were found by Iggo (1957) in the mucous membrane of the cat stomach. Failure to obtain motility responses in the forestomach of sheep with solutions in the pH range 7·9–10·1 does not rule out the presence of alkali-sensitive receptors. If such endings are present in the forestomach, either they do not affect motility or the keratinized epithelium is impermeable to the solutions used in these experiments or their threshold is higher than pH 10.

SUMMARY

1. The forestomach of the conscious sheep continued to contract after removal of the digesta. In most preparations the frequency of the contractions was within the range recorded in normally fed sheep.

2. Buffered solutions of acetic, propionic and butyric acid (100–200 mM) at pH 3·6–5·0 inhibited contractions, caused a marked fall in the pH value and a large increase in the concentration of steam-volatile fatty acid in the carotid blood. The onset of inhibition was not directly related to these changes.

3. Non-fatty acid buffers in a similar pH range and concentration did not inhibit, nor did they produce rapid and marked changes in pH of the carotid blood.

4. Fatty acid vapour inhibited contractions without producing large changes in the blood, although buffered solutions applied to localized areas of the forestomach failed to inhibit contractions.

5. The reticulum and rumen contracted in response to stimulation of the vagus nerve during acid inhibition.

6. Buffered solutions of phosphate, glycine-NaOH and rumen liquor made alkaline with sodium carbonate in the pH range 7·9–10·1 inhibited slightly or not at all.

7. It is concluded that the effects obtained with acid solutions were not simply a local pH effect but were related to the rapid penetration of free fatty acid through forestomach epithelium.

8. Since inhibition was produced by fatty acid vapour with only minor changes in the pH value, concentration of total CO_2 and steam-volatile fatty acid of the blood, inhibition seems to be due to peripheral stimulation of acid-sensitive receptors in the forestomach rather than to central nervous effects of chemical changes in arterial blood.

I wish to thank Dr A. T. Phillipson for his advice during the course of this work and Messrs J. Dunnett and L. Vowles for technical assistance.

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SOME OBSERVATIONS ON THE CHEMICAL COMPOSITION OF HORSE SWEAT

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It has long been known that horse sweat has a relatively high content of albumin (Leclerc, 1888; Smith, 1890) and a small quantity of globulin (Smith, 1890). Other compounds of protein character are not mentioned by these authors.

The presence of mucoprotein in human thermal sweat has been reported elsewhere (Jirka & Kotas, 1957), and Evans, Nisbet & Ross (1957) have demonstrated histochemically the presence of mucoprotein in horse sweat glands. It was therefore decided to look for mucoprotein in the sweat of the horse resulting from the exertion of racing. The opportunity has also been taken, in one instance, to analyse sweat resulting from emotion and to compare it with that after physical exertion of the same animal. Attention has been paid particularly to the concentrations of protein and electrolyte in the sweats.

In this paper mucoprotein is accepted as a protein substance of which about 10% consists of hexosamine.

METHODS

The sweat of three race-horses (mares) was collected after physical exertion and that of another mare, which sweated copiously immediately before a race, was collected both after pre-race emotion and after exertion. The sweat, which formed a foam, was scraped from the neck of the animal immediately after a race. On the occasion when emotional sweat was collected the animal's neck was dried before it ran. On each occasion a dirty brown fluid was obtained, which was freed from impurities (hair, dust, grains of sand) by centrifugation; after being filtered the fluid remained strongly opalescent or turbid. On the addition of sulphosalicylic acid a copious precipitate occurred.

Total nitrogen was estimated by the micro-Kjeldahl method. The nitrogen content of the filtrate, obtained after removal of proteins by adding to the sweat an equal volume of 20% sulphosalicylic acid, is referred to here as 'non-protein nitrogen'. This filtrate has been shown to contain mucoprotein. The precipitated protein calculated from the nitrogen difference is, on the basis of examination of the sweat by electrophoresis, accepted as albumin and globulin.

The presence of mucoprotein was polarographically investigated by Brdička's filtrate reaction, without alkaline denaturation, using the usual cobalt solution (Březina & Zuman, 1956). The sweat was deproteinized as stated and the concentration of mucoprotein, represented by the height of the polarographic wave obtained with the sulphosalicylic acid filtrate, was determined by comparison with a calibrating curve obtained with mucin isolated from the ox submaxillary gland according to the method of Blix (1936). The polarographic apparatus as well as the technique used has already been described (Jirka & Kotas, 1957).

In hydrolysed sweat hexosamine was determined according to the method of Elson & Morgan (1933) modified by Blix (1948) and adapted for small concentrations of mucoprotein by Kočent & Bošková (1957). The extinctions were measured at a wave-length of 530 mμ with a Beckmann spectrophotometer, model DU.

For both sweats, one associated with exercise and the other with emotion, (i) proteins were also estimated electrophoretically using Whatman No. 1 paper in a lucite chamber with barbital–citrate–oxalate buffer of pH 8·6 and ionic strength of 0·07; (ii) sodium and potassium were determined with a Zeiss flame photometer and (iii) chloride was determined by the method of Van Slyke & Hiller (1947).

RESULTS

The total and non-protein nitrogens as well as the combined albumin and globulin in the sweats of three horses after muscular exertion are given in Table 1. Sulphosalicylic acid filtrates of horse sweats were found to give the catalytic double wave in cobalt solution, when examined polarographically, which is characteristic of the presence of mucoprotein. The height of the polarographic wave obtained with the deproteinized sweat was about 70% of that obtained with sweat which was not deproteinized but was diluted with physiological saline in place of sulphosalicylic acid.

TABLE 1. The total nitrogen, non-protein nitrogen and albumin and globulin of horse sweat after physical exertion

Sample of sweat	Total N (mg/100 ml.)	Non-protein N (mg/100 ml.)	Albumin and globulin (g/100 ml.)
1	447	116	2·06
2	531	85	2·79
3	539	137	2·51

TABLE 2. The hexosamine and mucoprotein of horse sweat after physical exertion

Sample of sweat	Hexosamine (mg/100 ml.)	Mucoprotein (in terms of mg mucin/100 ml.)
1	46	125
2	113	135
3	84	100

TABLE 3. The composition of horse sweat resulting from emotion (*A*) and from physical exertion (*B*)

Sample of sweat	Total N (mg/100 ml.)	Non-protein N (mg/100 ml.)	Albumin and globulin (g/100 ml.)	Mucoprotein (in terms of mg mucin/ 100 ml.)	m-equiv/l.		
					Na	K	Cl
A	673	128	3·40	165	432	141	—
B	199	77	0·76	40	382	48	432

The hexosamine and mucoprotein contents are given in Table 2, and in

Table 3 the composition of the sweat collected during emotion is compared with that for the same animal after physical exertion. Electrophoretic analysis of the emotional sweat as well as of that after exertion showed mostly a protein portion with a mobility corresponding to human albumin at pH 8·6. Preceding the albumin fraction a small quantity of another fraction was observed, especially in the emotional sweat. In the sweat after exertion the mobility of this fraction was smaller; its spot interfered with the spot of albumin. After oxidation by periodic acid both fractions gave a positive leucofuchsin reaction. In the sweat after exertion traces of α_1-, α_2-, β- and γ-globulins were also detected.

DISCUSSION

The results of Leclerc (1888) and of Smith (1890), who found albumin and globulin in horse sweat, have been confirmed. According to the results of paper electrophoresis albumin is the chief protein; globulins are present only in traces.

The results of Brdička's filtrate reaction has proved the presence of mucoprotein in horse sweat. It is believed that the catalytic effect of proteins in cobalt solution, exhibited polarographically in sulphosalicylic acid filtrate, is due to the presence of —SH and —S—S— groups of mucoprotein. The polarographic reaction with solutions that have not been deproteinized would seem, in general, to depend on the presence of these groups in protein.

It is probable that the presence of hexosamine in hydrolysed sweat is derived especially from mucoprotein but it might possibly come from other hexosamine-containing compounds. In this connexion it is interesting that the relationship between the heights of the catalytic waves in polarographic records obtained with sulphosalicylic acid blood filtrates and the hexosamine contents of the same filtrates is approximately linear. This is believed to depend on the presence in blood serum of mucoprotein soluble in sulphosalicylic acid (Kočent, Brada & Bošková, 1957).

The electrolytes and total and non-protein nitrogen were more concentrated in the sweat resulting from emotion than in that from physical exertion. This is interesting; we should expect the sweat collected after physical exertion to be more concentrated by evaporation during the race. The differences in composition of the two samples of sweat are difficult to explain. According to investigations of Evans, Smith & Weil-Malherbe (1955; 1956) and of Bell & Evans (1956) the sweat glands in the horse are controlled by adrenaline. The adrenaline secretion increases in exertion (Evans *et al.* 1955) as well as in emotion. The question remains whether the mechanism of sweat secretion is different in the two conditions, or whether quantitative differences in adrenaline secretion could offer an explanation; or, again, whether as has been suggested to us by Sir Charles Lovatt Evans the differences in the composition of the two sweats are due to difference in skin circulation.

SUMMARY

1. Mucoprotein has been found in horse sweat. Albumins are confirmed as the chief protein.

2. Sweat caused by emotion contains higher concentrations of protein and electrolytes than does that after physical exertion.

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THE HYPOTHALAMUS AND SEXUAL MATURATION IN THE RAT

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Relatively little is known about the processes that underlie the onset of puberty, although it is apparent that the determining factors do not reside in the gonads or hypophysis. It is well established that immature ovaries show accelerated development when transplanted into an adult host (Foà, 1900) or after administration of gonadotrophic substances to infantile animals (Ascheim, 1926; Zondek, 1926). The pituitary glands of infantile rats grafted under the median eminence of the hypothalamus of hypophysectomized adults may rapidly function as mature glands, i.e. secrete gonadotrophic hormones at a time when the donor animals would still have been immature (Harris & Jacobsohn, 1952). Since the gonadotrophic functions of the adenohypophysis in adult animals have been shown to be dependent upon intact connexions between this gland and the hypothalamus (Benoit & Assenmacher, 1955; Donovan & Harris, 1955) it is reasonable to suggest that the onset of puberty is likewise controlled through the hypothalamus. Clinical workers (Dott, 1938; Weinberger & Grant, 1941; Lange-Cosack, 1951, 1952; Bauer, 1954) have found that precocious puberty in children is often associated with destructive processes in the posterior division of the hypothalamus.

The present study is concerned with the influence of hypothalamic lesions upon the time of puberty in rats. Vaginal canalization, which serves as an index of ovarian maturation, was found to occur significantly earlier in animals with lesions in the anterior part of the hypothalamus than in blank-operated controls or in rats with lesions in the pre-optic region. Part of the material to be described has been published in abstract form (Donovan & van der Werff ten Bosch, 1956; van der Werff ten Bosch & Donovan, 1957) and supplied the foundation of a more comprehensive unpublished essay which gained a Ciba Foundation Award for Basic Research Relevant to the Problems of Ageing in 1957.

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METHODS

The rats employed in this investigation were studied in four groups in the course of four experiments. The first experiment was performed in London, the subsequent three in Leiden. In the initial study infantile females of an inbred Lister strain were employed; later work was carried out on an inbred albino stock. All were obtained from the first litters of young females. The subjects of the first experiment were born in February 1956 and were operated upon when 14 or 15 days of age. After the surgical procedure they were returned to the mother together with their similarly operated brothers. These rats were housed in a blacked-out room artificially lit for eighteen hours a day and were fed on the Medical Research Council diet 41 and water. The rats used in Expt. 2 were born in October and November 1956, and were operated upon at 14 days of age, the rats of Expt. 2*A* were born in November and December 1956 and operated upon when 14 days old: those of Expt. 3 were born in April and May 1956 and operated upon at 10 days of age. On the day of operation the females from two litters were combined and put with one doe; the other reared the discarded males. The rats of Expts. 2, 2*A* and 3 were exposed to scanty daylight and artificial illumination for 8 hours daily and were fed on a standard comprehensive diet and water.

All operations were performed under ether anaesthesia. The lesions were placed with the aid of a stereotaxic machine, fixation of the head being obtained by means of ear plugs and a mouth bar and clamp over the nose. A short incision in the skin of the head was made in the mid line along the sagittal suture of the skull. A platinum-wire glass-insulated electrode with 1 mm bare tip was then lowered through the sagittal suture and the sagittal sinus to the floor of the hypothalamus. Anodal lesions were made by passing 2 mA d.c. for 15 sec, using an indifferent electrode inserted into the rectum. Blank-operated animals differed from their litter-mates only in that no current was passed when the electrodes were in position. Blood loss was always negligible, amounting only to a single small drop. The members of a litter were divided between the operated and blank-operated groups by alternating between the two procedures.

The infantile rats were returned to the mother about 1 hr after operation. They were then examined daily for vaginal opening and weighed twice weekly. In a number of cases vaginal smears and reproductive performance were studied after vaginal canalization and in a few instances the effect was examined of subcutaneous progesterone administration on the vaginal smears. The rats were killed with chloroform or ether, the uterus, ovaries and adrenals were removed and weighed fresh and the brain fixed in 10% formalin after removal of the calvarium. The organs except the brain were embedded in paraffin and 7 or 10μ sections were stained with haematoxylin-eosin. The brains were embedded in low-viscosity nitrocellulose, cut serially at 100μ and stained with Weigert's iron haematoxylin (Expt. 1) or embedded in paraffin, cut at 10 μ, and every tenth section mounted and stained with Luxol Fast Blue and cresyl violet (Klüver & Barrera, 1953) (Expts. 2, 2*A* and 3).

Data for unoperated rats were derived from 26 animals of the Lister strain (born March and April 1956) and from albino rats born in April 1956 (22 rats) and in November 1956 (54 rats).

RESULTS

Experiment 1. *Lister rats operated upon at* 14 *or* 15 *days of age and killed* 20 *days later*

Onset of puberty (Fig. 1). Twenty-six normal rats of this strain were studied in order to determine the age at which vaginal canalization normally occurred. The average age at this event was 40·9 ± 0·7 (S.E.) days.

The experimental rats consisted of eight operated animals and nine blank-operated. The operations were carried out at 14 or 15 days of age and all rats were killed when one member of the blank-operated group was found to possess an open vagina. This rat was then 34 days old and had been operated upon 20 days earlier. At this time vaginal opening had already occurred in all the operated rats. The earliest age at puberty was 30 days (two rats) and the average age 32·6 ± 0·7 days.

Location of lesions. In five rats (nos. 1, 18, 20, 22 and 25) the lesions were placed immediately behind the optic chiasma, extending upward from the floor of the hypothalamus over a distance of about 1 mm and just involving

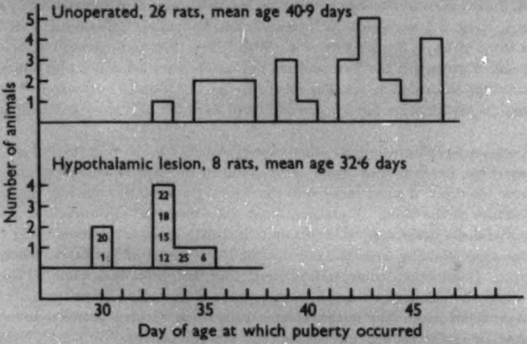

Fig. 1. Diagram to illustrate the advancement of the onset of puberty produced by a hypothalamic lesion in Expt. 1. In this and in Figs. 2, 3 the height of the block indicates the number of animals which reached puberty on each day. The numerals within the blocks identify individual animals.

the basal portion of the paraventricular nuclei. In three other animals (nos. 6, 12 and 15) the lesions lay more anteriorly, above the optic chiasma and reaching upward toward the anterior commissure. The lesions were not invariably in the mid line, unilateral lesions being found in nos. 6, 12, 15 and 25. The hypothalamus was normal in all the blank-operated animals.

Organ weights and gonadal function. There was a striking difference between the weights of the uteri of the operated and the blank-operated rats. The uterine weights of the former group ranged from 75 to 139 mg (mean 96·9 ± 9·2) and for the eight infantile blank-operated animals from 21·5 to 46 mg (mean 34·0 ± 2·9). Thus the occurrence of vaginal opening provided a true index of the presence of active gonads. The ovaries were slightly, but not significantly, heavier in the operated series (mean 19·9 ± 1·4 mg) than in the eight infantile controls (mean 15·6 ± 1·2 mg) and ovulation had occurred in six of the rats in which the onset of puberty was hastened (nos. 1, 12, 18, 20, 22 and 25), but in none of the controls. Body weights and adrenal weights did not vary significantly between experimental animals (body weight: mean

62·6 ± 1·9 g; adrenal weight: mean 20·1 ± 1·6 mg) and controls (body weight: mean 60·7 ± 3·0 g; adrenal weight: mean 19·3 ± 1·5 mg).

Experiment 2. *Albino rats operated upon at* 14 *days of age*

Onset of puberty in unoperated and blank-operated animals. The data for these rats are presented in Fig. 2 and Table 1. In this experiment fifty-four intact rats born in November 1956 were studied to obtain information on the normal time of vaginal opening. Puberty in this group was reached at an average of 46·1 days (range 36–64 days). Twenty rats were subjected to a blank operation at 14 days of age and puberty occurred some 23 days later (average: 37·2 days of age; range 32–43 days). Thus in this experiment the surgical intervention appeared to advance the onset of puberty.

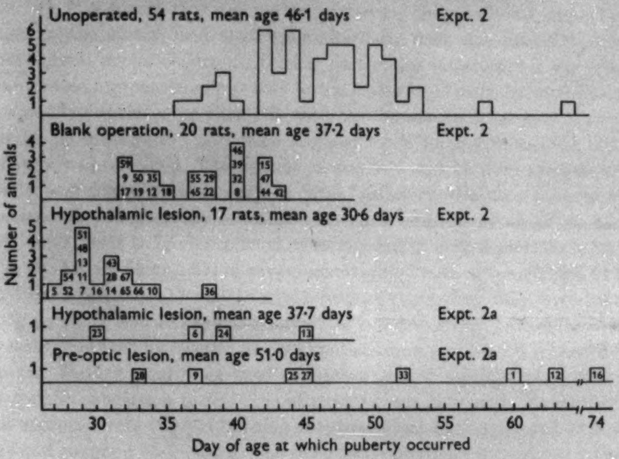

Fig. 2. Age at puberty in Expts. 2 and 2A. Plotting as in Fig. 1.

TABLE 1. Mean age at puberty, and standard error, of the albino rats of Expts. 2, 2A and 3

	Season born							
	Autumn				Spring			
Operation	Expt.	No. of rats	Mean age (days)	S.E.	Expt.	No. of rats	Mean age (days)	S.E.
Unoperated	2	54	46·1	0·7	3	22	40·3	0·6
Blank-operated		20	37·2	0·9		34	43·3	0·8
Lesion in hypothalamus		17	30·6	0·7		13	38·1	1·3
Lesion in hypothalamus	2*A*	4	37·7	3·2				
Pre-optic lesion		8	51·0	4·9				

Onset of puberty in operated animals. Seventeen animals with hypothalamic lesions were studied in this series. Twenty of their litter-mates were subjected to blank operations (see above, Fig. 2 and Table 1). All were born in October 1956. In the animals bearing hypothalamic lesions vaginal opening occurred at an average age of 30·6 days (range 27–38 days), and was therefore markedly advanced in comparison with the normal controls (46·1 days) or blank-operated animals (37·2 days). Actually, sixteen of seventeen operated animals had reached puberty before any of fifty-four unoperated rats, and at a time when only seven of twenty blank-operated rats displayed vaginal canalization.

TABLE 2. Summary of hypothalamic structures damaged by the lesions of Expts. 2 and 2*A*. In Expt. 2 the rats are arranged in the order in which they reached puberty

Rat no.	Optic chiasma	Anterior commissure	Supra-chiasmatic nuclei		Supra-optic nuclei	Fornix	Paraventricular nuclei	Ventromedian nuclei
Expt. 2								
5	+ +	.	+ + +	.	+	+ + +	.	.
52	+ +	.	+ + +	+ + +	.	.	.	.
54	+ + +	.	.	.	+ + +	+ + +	+ +	+ + +
7	+ +	+ + +	+ + +	.	.	.	.	.
11	+ + +	+	+ + +	+ + +	+	.	.	.
13	+ + +	.	+ + +	+ + +	.	.	.	.
48	.	.	.	.	+ + +	+ + +	+ + +	+ + +
51	+ +	.	+ + +	.	.	.	+	+ + +
16	+ + +	.	+ + +	+	.	.	.	.
14	+ + +	.	+ + +	+ + +	.	.	.	.
28	+ + +	+ + +	+ + +	+ + +	+ +	+ + +	.	.
43	+ +	+ + +	+ + +	+ + +	.	+ + + + + +	.	.
65	+ + +	+ + +	+ + +	.	.	.	.	.
67	+ + +	+ +	+ + +	+ + +	.	.	.	.
66	+	+ + +	.	.	.	.	.	.
10	+ + +	.	.	.	.	.	.	.
36	+ + +	+ + +	+ + +	+ + +	.	.	.	.
Expt. 2*A*								
6	.	+ + + + + +	.	.	.	+ + +	.	.
13	+	+	.	.	+	+ + + + + +	.	.
23	.	+ + + + + +	.	.	.	+ + +	+ + +	.
24	.	+ + + + + +	.	.	.	+ + +	.	.

+, slightly damaged; + +, markedly damaged; + + +, completely destroyed. Where a bilateral structure was injured on both sides, this is indicated by two sets of symbols.

Location of lesions. The hypothalamic region of the brain was studied histologically in all animals. The lesions varied in size and precise localization and their positions are summarized in Table 2. This table shows that the optic chiasma and suprachiasmatic nuclei were partly or wholly destroyed by nearly all the lesions. Most of the animals must have been blind, as judged from the histological appearance of the optic system. Dilatation of the third ventricle was observed in the majority of animals. Although the precocious onset of puberty cannot be correlated with damage to any specific hypothalamic component, the operated area in this group was in the anterior hypothalamus, between the optic chiasma and the pituitary stalk.

Ovarian function. Reproductive function was studied in seven blank-operated and seven operated rats. Normal oestrous cycles, as indicated by vaginal smears, normal fertility and undisturbed lactation were found in seven blank-operated rats (nos. 8, 9, 12, 15, 17, 18 and 19) and in three operated rats (nos. 10, 11 and 16). Four operated rats demonstrated prolonged oestrus, i.e. cornified vaginal elements in over 60% of the daily smears and of these one (14) was fertile, whereas the other three (nos. 5, 7 and 13) failed to become pregnant when left with a series of males over a number of weeks. It was found that 0·1 mg progesterone daily induced some cyclic changes in the smear, and that when the daily dose was raised to 0·5 mg almost normal cycles occurred. During part of the latter period the progesterone-treated animals were put with fertile males for 17 days, but none became pregnant. The males were then left with the female operated rats after progesterone administration had been stopped for a further 4 months but no pregnancy was obtained. The ovaries of rats 5, 7 and 13 were studied histologically and found to lack corpora lutea, although Graafian follicles were abundant. A wider survey revealed that such ovaries, in which no recent ovulations appear to have occurred, were present in eleven (nos. 5, 7, 11, 13, 16, 28, 36, 43, 48, 52 and 67) of seventeen operated rats, although rats 11 and 16 had been pregnant 13 and 18 weeks respectively before they were killed.

Body weight at time of vaginal opening. It is possible that hyperphagia may have been produced by a lesion in the hypothalamus, and since it is known that improved dietary conditions tend to hasten sexual maturation a study of body weight in relation to the time of puberty was indicated. In the twenty blank-operated rats vaginal opening occurred when they averaged 37·2 days of age and weighed 95·7 ± 2·9 g. The seventeen operated rats reached puberty at an average age of 30·6 days and weighed 68·9 ± 2·0 g. When the blank-operated rats were about 31 days old they were somewhat heavier, 74·3 ± 2·0 g. Early puberty was therefore not a consequence of an increased rate of bodily development.

Age, body weight and organ weights at death. The operated rats were killed in three groups, 7–10, 27–32, and 52 weeks after birth. The data are summarized in Table 3. At the age of 7–10 weeks the operated rats were significantly smaller ($P < 0{\cdot}01$) than blank-operated litter-mates, and their ovaries were likewise smaller ($P = 0{\cdot}05 - 0{\cdot}02$), even when expressed in mg/100 g body weight. A main cause of the lower average ovarian weight in the operated animals seems to be the absence of corpora lutea in a large proportion of them. By contrast, the uterine weight of operated animals was larger ($P < 0{\cdot}01$)

than that of the blank-operated group, possibly owing to longer phases of oestrus. After 27–32 weeks the differences in body, ovarian and uterine weights were no longer significant. There were no significant differences in the weights of the adrenal glands. None of the animals younger than 10 weeks had been pregnant or treated with hormones.

Experiment 2A. Albino rats operated upon at 14 days of age, with lesions intended for the pre-optic area

Onset of puberty (Table 1, Fig. 2). The twelve rats employed in this study were born in November and December 1956. At post-mortem four of these were found to have lesions in the anterior part of the hypothalamus (Table 2); they reached puberty at an average age of 37·7 days (range 30–45 days, Fig. 2). In eight rats with a lesion in the pre-optic area, the average age on the day of vaginal opening was 51·0 days (range 33–74 days). In comparison with the unoperated rats of Expt. 2 which were born at about the same time, puberty was not advanced in the animals with lesions in the pre-optic region.

TABLE 3. Body and organ weights of the rats in Expts. 2, 2*A* and 3. The figures have been grouped according to the age when killed

Expt.	Group	Age (weeks)	No. of rats	Body weight (g) (mean ± S.E.)	Weight (mg/100 g body wt., mean ± S.E.)		
					Ovaries	Uterus	Adrenals
2	Blank-operated	8–10	13	149 ± 7	34·7 ± 2·3	126 ± 8	24·9 ± 1·4
		32	7	275 ± 7	24·8 ± 2·0	202 ± 21	21·6 ± 1·6
	Operated	7–10	10	117 ± 6	26·7 ± 3·1	187 ± 15	22·1 ± 2·1
		27–32	4	248 ± 18	19·8 ± 1·4	220 ± 27	18·0 ± 1·1
		52	3	250 ± 12	21·3 ± 3·0	131 ± 20	19·3 ± 1·1
2*A*	Pre-optic lesion	22–25	7	195 ± 9	38·4 ± 1·5	134 ± 8	24·0 ± 0·6
3	Blank-operated	39–42	31	261 ± 6	26·0 ± 2·1	173 ± 8	18·9 ± 1·2
	Operated	39–42	12	233 ± 12	25·3 ± 3·6	158 ± 16	17·7 ± 1·9

Location of lesions. The lesions in the pre-optic region lay more than 0·5 mm anterior to the anterior commissure and were bilateral, except in number 9. They were located in the frontal lobes and in several cases impinged upon the olfactory bulbs; they did not touch the base of the brain. The anteroposterior extension of the lesions was 1–2 mm. The hypothalamus and optic system remained intact. The lesions in the hypothalamus (see Table 2) were situated in the mid line in three animals. Numbers 6, 23 and 24 had each a high lesion in and extending behind the anterior commissure, which in number 6 reached down to the base of the brain. Rat 13 had a fairly large lesion which began in front of the anterior commissure and reached down to the posterior end of the supraoptic nucleus on one side; on the other side a small lesion severed the fornix immediately behind the anterior commissure.

Ovarian function. All animals were caged with males for three weeks. Pregnancy and delivery of litters occurred in two rats with lesions in the hypothalamus (nos. 6 and 13) and in four rats with pre-optic lesions (nos. 1, 9, 12 and 16).

Age, body weight and organ weights at death. The data for seven animals with pre-optic lesions, which were killed 22–25 weeks post-partum, are summarized in Table 3. In comparison with the other groups the body weights seem lower than might have been expected at the age of 22–25 weeks, and the rather large size of the ovaries and adrenals (in mg/100 g body weight) is probably due to this fact; the average uterine weight was small.

Experiment 3. *Albino rats operated upon at* 10 *days of age*

Onset of puberty (Table 1, Fig. 3). This experiment employed seventy-one animals. The average age on the day of vaginal opening in twenty-two unoperated rats born in April 1956 was 40·3 days (range 33–45 days), and the corresponding figure for thirty-four blank-operated rats (litter-mates of those operated on, and born in May 1956) was 43·3 days (range 33–52 days). The difference is statistically significant ($P < 0{\cdot}01$) and it thus appears that in this experiment the blank-operation delayed puberty. Comparison with Expt. 2 (Table 1) indicates that puberty occurred significantly earlier ($P < 0{\cdot}01$) in the unoperated rats born in early summer than those arriving later in the year.

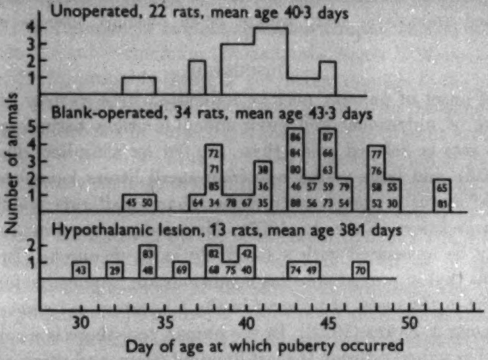

Fig. 3. Ages at puberty of the rats in Expt. 3. Plotting as in Fig. 1.

Hypothalamic lesions were placed in thirteen rats. Vaginal opening in this group occurred at ages between 30 and 47 days, with an average of 38·1 days. It is apparent that the time of vaginal canalization has been significantly advanced ($P < 0{\cdot}01$) by the hypothalamic lesions in the 10-day old rats of this experiment, in comparison with the blank-operated litter-mates.

Location of lesions. On the whole, the lesions appeared very small. This is perhaps largely due to the long survival time; nearly all the rats in this experiment were killed at the age of about 40 weeks. Lesions immediately behind the optic chiasma, largely consisting of a basal lesion with thickening of the floor and irregular outlines of the lumen of the third ventricle, were found in six rats (nos. 42, 70, 74, 75, 82 and 83). Five rats (nos. 29, 40, 48, 49 and 68) had each a lesion in the infundibular region which did not involve the pituitary stalk. One rat (no. 69) had a lesion directly in front of the mammillary bodies and in a further rat (no. 43) the lesion was situated behind the mammillary bodies.

Ovarian function. Vaginal smears were studied in six operated and in fourteen blank-operated rats. The cycles were normal in all. Pregnancy occurred in all five operated and thirteen blank-operated animals which had been with males.

Age, body weight and organ weights at death. Nearly all animals were killed at approximately 40 weeks of age. The summarized data appear in Table 3 and it may be seen that no significant differences existed between blank-operated and operated animals in body weight or in ovarian, uterine and adrenal weights.

DISCUSSION

The time of onset of puberty may be influenced by a variety of factors, of which those of nutritional and environmental origin assume prominence. Puberty in rats is delayed when these are fed on a limited diet (Asdell & Crowell, 1935), and large young rats from small litters have been noted to reach sexual maturity significantly earlier than small rats which had been reared in large litters (Kennedy, 1957). Moreover, in the human, childhood obesity may be associated with a relatively early menarche (Bruch, 1941). It is probable that improved breeding conditions are responsible for the steady advancement in the onset of puberty in the laboratory rat since the pioneer studies of Long & Evans (1922). In the human too, there is a marked trend towards earlier puberty during the last hundred years, and this is perhaps due, likewise, to a general improvement in environmental circumstances and nutrition (Tanner, 1955). Environmental stimuli acting through the nervous system modify the timing of sexual maturation, for it has been found that in infantile rats subjected to constant illumination vaginal opening was hastened (Luce-Clausen & Brown, 1939; Fiske, 1941), and the first oestrus occurred earlier (Jöchle, 1956) in comparison with animals kept under normal laboratory conditions. Conversely, in rats reared in darkness the onset of puberty has been found to be delayed. Seasonal factors, as yet unidentified, are also of importance. In the present work it has been found that a group of normal rats born in April reached puberty significantly earlier than animals born in November. Such observations underline the necessity of incorporating littermate control animals in any experiment to analyse the factors governing the onset of puberty.

It has been suggested (Mandl & Zuckerman, 1952) that non-specific 'stress' may raise the output of gonadotrophic hormone by the pituitary gland and thus advance the onset of puberty in rats. This view is based on the finding that the average time of vaginal opening (35·1 days) was advanced by 3·5 days in thirty-eight rats which had been repeatedly exposed to cold. In addition, Mandl & Zuckerman found that 'rough-handling' delayed the average time of vaginal opening for 3 days; the average age at puberty of 36 'rough-handled' rats being 41·6 days as compared with 38·7 days for the thirty-four rats serving as controls in the cold-exposure experiment. It would seem, therefore, that only particular forms of stress hasten puberty and that the term 'non-specific stress' is not applicable. In the present work, in Expt. 2 blank operation appeared to advance puberty by 8 days and in Expt. 3 to delay it by 3 days. Control and blank-operated groups were not, however, composed of litter-mates and so no conclusions can be drawn.

The effect of hypothalamic lesions on the onset of puberty has seldom been studied. In experiments on rabbits, gonadal atrophy ensued when lesions were placed in the tuber cinereum (Bustamante, Spatz & Weisschedel, 1942; Bustamante, 1943), probably owing to concurrent damage to the hypophysial portal system, whilst the experiments of Gaupp (1950) on the same species are of doubtful significance. In three experiments of the present study puberty was advanced beyond doubt in rats operated upon at 10, and at 14 or 15 days of age. Confirmatory findings in the rat have since been made by E. M. Bogdanove (personal communication). An action of the lesion upon gonadotrophin secretion is indicated by the observation that in Expt. 1 the uterine weights of the operated animals were much above those of their blank-operated controls and that six of the nine had ovulated. The last finding rules out the possibility of an adrenal factor being involved in the response.

It has not proved possible to associate damage to any particular hypothalamic structure with the advancement of puberty. Rather there appears to be a fairly well-defined area behind the optic chiasma which is concerned in the response. On the other hand, puberty is, if anything, delayed by electrolytic damage to the pre-optic area. These findings in the rat differ from those reported in connexion with clinical cases of precocious puberty. In children with sexual precocity accompanied by pathological processes in the hypothalamus, the more localized malformations, such as tumours or hamartomas, are usually situated in the posterior part of the hypothalamus, close to or involving the mammillary bodies. Experimentally effective lesions in the rat were all positioned in the anterior hypothalamus. No explanation can yet be offered for this discrepancy.

The mechanisms underlying the gonadal response to hypothalamic damage are as yet obscure, although a direct action on the hypothalamic–pituitary–gonadal axis is indicated. An indirect effect derived from a modification in growth rate due to hyperphagia might be presumed. However, hyperphagia was not observed and in Expt. 2 the body weight of operated rats at the time of vaginal opening was, if anything, less than that of blank-operated rats of the same age, which reached puberty six to seven days later. Moreover, hyperphagia has been commonly associated with destruction of the ventromedian nuclei and these were almost uniformly intact.

Any explanation for the finding that lesions in the anterior part of the hypothalamus are followed by precocious onset of puberty should take into account the information available with regard to the pituitary-gonadal relationship in the infantile animal. The mode of control of the adult sexual cycle should also be taken into consideration, for it is reasonable to suppose that a similar mechanism operates during both phases of life. There is no doubt that the infantile pituitary gland secretes gonadotrophic hormone, for hypophysectomy of infantile rats results in an arrest of gonadal development both in females (Walker, Simpson, Asling & Evans, 1950) and in males (Walker, Asling, Simpson, Li & Evans, 1952). The infantile gonad likewise produces sex hormones: prepubertal castration is followed by early regression of male accessories in the rat (Clark, 1935), by increased excretion of gonadotrophin in a child (Hamilton, 1948), by an increase in pituitary content of gonadotrophin (Hellbaum & Greep, 1940), by an increase in pituitary weight (van Rees & Paesi, 1955) and by the development of 'castration-cells' in the pituitary gland (Dohrn & Hohlweg, 1931). Also, if one of a pair of infantile rats united in parabiosis is castrated, then the partner will display precocious puberty (Kallas, 1929, 1930). Such changes following gonadectomy in the infantile individual are believed to be due to a lack of gonadal hormone in the blood and, indeed, it has been demonstrated that castration changes in the pituitary gland of infants can be prevented or inhibited by exogenous gonadal hormone (Dohrn & Hohlweg, 1931), whereas precocious puberty in an otherwise intact parabiotic rat can be prevented by the administration of low doses of gonadal hormone to the castrated partner (Byrnes & Meyer, 1951). A temporary fall in the blood level of gonadal hormone, with a consequent increase in gonadotrophin secretion, is believed to be the cause of the precocious puberty which is observed after auto-transplantation of infantile ovaries (Pfeiffer, 1936; Greep & Jones, 1950; Almqvist & Jacobsohn, 1956; Donovan & van der Werff ten Bosch, unpublished). On the basis of the above evidence it would seem that the feed-back action of gonadal hormones upon the pituitary gland, which was postulated by Moore & Price (1932) to account for the rhythmical nature of the ovarian cycle in the adult, is also apparent during infancy.

In the adult, it is now clear, the feed-back action of gonadal hormone is exerted, not directly on the pituitary gland, but on the hypothalamus (see Donovan & Harris, 1959). A variety of studies indicate that the consequences of lesions in the anterior hypothalamus of the rat (suspension of ovulation, constant oestrus) follow from interference with a feed-back action of oestrogen exerted in this region (Flerkó, 1954; Flerkó & Szentágothai, 1957). It is also possible that a similar servo-mechanism operates during infancy. The most attractive explanation for the present findings would be that damage to or destruction of the gonadal feed-back area in the hypothalamus initiates the secretion of gonadotrophin in amounts sufficient to promote the onset of sexual maturation. An important difference between infantile and adult individuals, which could account for the relative quiescence of the infantile gonad, would appear to be a higher sensitivity of the infantile hypothalamus to gonadal hormone. Hohlweg & Dohrn (1931) found that in infantile rats the pituitary changes after gonadectomy could be prevented by gonadal hormone in doses approximately one-hundredth of that required in the adult. A fundamental change, then, in the advance toward sexual maturity would seem to be a reduction in the sensitivity of the hypothalamus toward gonadal hormone. On the basis of the information outlined above, it is postulated that in the present work (*a*) the onset of puberty has been hastened by release of the pituitary gland from an inhibition exercised by the central nervous system through the mediation of the anterior part of the hypothalamus, and (*b*) that the release is possibly brought about by a blockade of the action of gonadal hormone upon the hypothalamus.

An alternative explanation for the findings described above is that the lesions have exerted an irritating and excitatory action upon the surrounding surviving nervous structures, perhaps through some material released by the damaged nerve tissue. It is difficult to refute this possibility, but attention may be drawn to the results of Donovan & van der Werff ten Bosch (unpublished) from a parallel study of the effects of hypothalamic lesions in the ferret where this matter has also received attention.

A number of workers have found that lesions placed in the hypothalamus immediately behind the optic chiasma may cause constant or prolonged oestrus in the guinea-pig (Dey, Fisher, Berry & Ranson,1940; Dey, 1943) and rat (Hillarp, 1949; Greer, 1953; Alloiteau, 1954). In the course of Expt. 2 it was found that some but not all of the sexually precocious rats likewise displayed prolonged oestrus and that, as in the experiments of Greer (1953) and Alloiteau (1954), progesterone would restore cyclic activity, though not fertility. It is tempting to speculate upon a mechanism common to the phenomena of precocious puberty and constant oestrus, but it should be emphasized that a number of the precocious rats had normal vaginal cycles and were fertile.

SUMMARY

1. The time of onset of puberty, as indicated by vaginal canalization, was studied in Lister and albino rats. Seasonal influences and the effect upon sexual maturation of blank-operations and of lesions placed in the hypothalamus were examined.

2. Unoperated albino rats born in spring reached puberty 5 days earlier than those born in autumn.

3. In comparison with blank-operated litter-mates, lesions placed in the anterior hypothalamus 14 days post-partum advanced puberty by 7 days, and lesions placed 10 days post-partum hastened puberty by 5 days.

4. Effective hypothalamic lesions were situated basally immediately behind the optic chiasma; lesions in the pre-optic region did not hasten the onset of puberty.

5. An action of the hypothalamic lesions upon gonadotrophin secretion is indicated, since the uterine weights of animals killed within a few days of vaginal canalization were much above those of their blank-operated controls and most of the operated rats had ovulated.

6. Effective lesions did not cause obesity.

7. Some rats with hypothalamic lesions displayed prolonged oestrus and their ovaries contained no corpora lutea.

8. In conclusion it is postulated that effective lesions released the pituitary gland from an inhibitory influence, possibly by a blockade of the feed-back action of gonadal hormone.

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THE RELATIONSHIP OF THE HYPOTHALAMUS TO OESTRUS IN THE FERRET

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It is now quite clear that many aspects of endocrine activity are regulated through the hypothalamus. In studies of these mechanisms, use has been made of the techniques of either stimulation or destruction of various areas of this part of the brain. Thus de Groot & Harris (1950) found that electrical excitation of the posterior region of the tuber cinereum of conscious rabbits produced a lymphopenia indicative of the secretion of adrenocorticotrophic hormone. Conversely, electrolytic lesions in this region interfered with the secretion of this hormone in response to emotional stress (de Groot & Harris, 1950). Similarly, Ganong, Frederickson & Hume (1955) found that electrolytic lesions in the anterior hypothalamus of the dog depressed the rate of thyroidal accumulation of radioactive iodine, and Harris & Woods (1956) found that electrical stimulation of the same region in the rabbit was associated with the accelerated discharge of protein-bound ^{131}I from the thyroid gland. Such effects are believed to follow alterations in the amount of thyrotrophic hormone released by the hypophysis.

When the secretion of the gonadotrophic hormones is considered the experimental findings are a little different. Stimulation of the hypothalamus has been found to induce release of pituitary hormone as evidenced by ovulation in the rabbit (see Donovan & Harris, 1959) and pseudopregnancy in the rat (Critchlow, 1957). However, many of the findings of regressive changes in the gonads following lesions in the hypothalamus may now be ascribed to inadvertent interference with the hypophysial portal vessels which supply the pituitary gland and not to hypothalamic damage *per se*. Nevertheless, modifications in the sexual cycle following hypothalamic lesions have been recorded on numerous occasions and sometimes there are indications that destruction of part of the hypothalamus may have resulted in *increased* gonadotrophin secretion. It is well known that certain lesions in the posterior region of the hypothalamus may promote the secretion of gonadotrophin and the precocious onset of puberty in children. Experimentally, lesions placed in the anterior hypothalamus of infantile rats have been shown to advance puberty (Donovan & van der Werff ten Bosch, 1956*a*, 1959). In the present work it has been found that anterior hypothalamic lesions in the anoestrous ferret advance the onset of the breeding season, as previously reported in abstract (Donovan & van der Werff ten Bosch, 1956*b*). This work, now extended to include ferrets with lesions in the posterior hypothalamus, the thalamus and the amygdaloid region, together with studies on the effect of electrical stimulation of the hypothalamus of the anoestrous ferret, is here presented in detail.

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METHODS

Female albino ferrets of mixed stock were used, with the exception of numbers 76, 78, 92, 181, 202, 238, 282, and 306, which were of the polecat variety. Some of the animals were born in the laboratory during the preceding summer but the majority were purchased shortly before the beginning of each experiment.

Lesions

The animals used in the studies with lesions were housed in a wooden hut lit by daylight supplemented by two 60-watt incandescent electric lamps between 9 a.m. and 5 p.m. They were fed on a diet of tinned dog food and fresh milk with the addition of cod liver oil and wheat germ oil. Water was constantly available.

The lesions were placed under general anaesthesia (pentobarbitone sodium, Nembutal; Abbott Laboratories; 36 mg/kg body weight, intraperitoneally) during the normal winter anoestrum. A stereotaxic machine was employed which held the head in such a position that the lowest point on the rim of the orbit and the axis of the ear plugs lay in the same horizontal plane; the axis of the ear plugs thus formed both a transverse and a horizontal zero. For anterior hypothalamic lesions a glass-insulated platinum wire electrode, with an uninsulated tip of 1 mm, was lowered at three points (mid line and 1 mm to each side of the mid line) 4 mm anterior to the transverse zero plane, to a depth of 1 mm above the horizontal zero, and anodal lesions were made by passing a direct current of 3 mA for 1 min at each point. The indifferent (cathodal) electrode was inserted into the rectum. For posterior hypothalamic lesions the electrode was inserted in the plane of the transverse zero 0·5 mm on each side of the mid line to a depth of 1 mm above zero. Amygdaloid lesions were made by inserting the electrode at three points on each hemisphere, 4 mm anterior to zero and 5 and 6 mm lateral to the mid line, and 5 mm anterior to zero and 5 mm lateral to the mid line. The electrode was inserted in each case to the depth of zero and 3 mA passed for 1 min. In the blank operations the above procedures were repeated exactly but the current was not passed. Blood loss was negligible in all cases. A mixture of streptomycin and penicillin was dusted into the wound before the skin was sutured.

Each ferret was weighed weekly; at the same time it was examined and the condition of the vulva noted. The onset of oestrus in the ferret is characterized by swelling of the lips of the vulva, which then enlarges to form a structure some 2 cm in diameter. At the end of the experiment the animal was killed with chloroform, the ovaries, uterus and adrenals removed and the head perfused with 10% formalin via the aorta. The thyroid glands were then dissected free and the brain and hypophysis removed from the skull. Each brain was embedded in low-viscosity nitrocellulose, serially sectioned and stained with Weigert's iron haematoxylin or Loyez's stain. The endocrine glands were stained with haematoxylin and eosin.

In a number of ferrets the uptake of ^{131}I by the thyroid gland was measured over a four-hour period using the technique described by Donovan & Harris (1956).

Stimulation of the hypothalamus

These experiments were carried out during the winters of 1954–55 and 1955–56. In the first series a unipolar electrode was used, consisting of a length of platinum wire (SWG 36) in an insulating glass capillary; the tip of the platinum wire was uninsulated for 1 mm. The electrode was inserted into the hypothalamus and fixed in a tapered metal bush screwed into the dorsal aspect of the skull. A stainless-steel plate attached to the temporalis muscles formed an indifferent electrode and leads from both electrodes were conducted beneath the skin to brass pins sealed in a Perspex carrier. The carrier was attached to the muscles of the lumbar spine and the pins, insulated by a ring of Perspex, emerged through the skin. In this way the hypothalamus could be stimulated electrically in the conscious animal by fitting a socket carrying light connecting wires to the plug on the animal's back. Alternatively, a coil composed of about 3000 turns of s.w.g. 42 enamelled copper wire could be fitted to the plug and the ferret stimulated by the remote control technique described by Harris (1947). Some ferrets were subjected to long periods of stimulation whilst housed in a ventilated but light-proof box. By this means the ferrets could be stimulated in complete darkness except for the few minutes daily when the box was opened for cleaning, inspection of the electrical connexions and the supply of food and water.

In the second series bipolar glass-insulated platinum wire electrodes were used which were fixed in position with dental cement. Leads from the electrodes were run subcutaneously to the thoracic region, brought out through an opening in the skin and attached to a leather harness. Before connexion to the harness and before suturing the skin, the leads were knotted together in such a way that traction exerted upon them would be transmitted to the skin and not to the electrodes in the head. The ferrets were kept in the laboratory but were kept in darkness by screening with light-proof curtains from about 4 p.m. to 9 a.m. daily, thus being exposed to 7 hr light per day. Stimulation was provided from the 50 c/s mains through a step-down transformer and variable resistance. A mercury switch was inserted into the secondary of the transformer so that a rhythm of 15 sec stimulation, 15 sec rest, 15 sec stimulation, 15 sec rest, etc., was maintained.

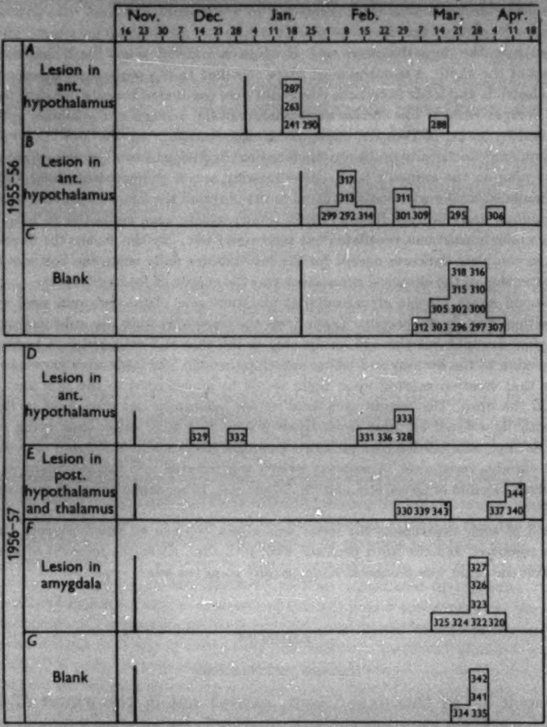
At the end of each experiment the ferret was killed, the genital tract, thyroid and adrenal glands were removed, and the head perfused with formalin. After the removal of the electrode from the skull the brain was processed histologically as in the lesion experiments.

RESULTS

Lesion experiments

Experiment 1. In this experiment, carried out in the winter of 1955–56, lesions intended for the anterior hypothalamus were placed in the brains of fifteen ferrets. Twelve ferrets were subjected to blank operations. The animals were prepared in two series, the second series being operated upon when the effect of the lesion on some of the first five ferrets was observed in January 1956.

Onset of oestrus. The date of operation and the time of onset of vulval swelling for each animal are indicated in Text-fig. 1 (*A*, *B*, *C*). It will be seen that eleven of the fifteen operated animals became oestrous before any of the twelve blank-operated ferrets. The latter, control, ferrets started to display vulval swelling in March, which is the normal time of year in England (see Donovan & van der Werff ten Bosch, 1956*c*). It is noteworthy that nine ferrets came into oestrus within 4 weeks of the placement of the lesion. This interval is similar to that sometimes observed in other experiments when ferrets were exposed to prolonged illumination (Donovan & Harris, 1956).



Text-fig. 1. Diagram to illustrate the effect of various operations on the brain on the onset of oestrus in the ferret. In this figure the height of the block indicates the number of animals which began to show vulval swelling each week. The numerals within the block identify individual animals. The animals marked by an asterisk possessed lesions confined to the thalamus. The solid vertical bars indicate the time of operation.

Position of lesions. Generally the lesions were situated basally in the anterior part of the hypothalamus, between the optic chiasma and the pituitary stalk, and extended upward to the level of the paraventricular nuclei (Pl. 1, figs. 1, 2). A detailed summary of the lesions is given in Table 1. It will be seen that the optic chiasma, the suprachiasmatic nuclei and the fornices are almost invariably involved. In most of the ferrets damage was also sustained by the supra-optic, the paraventricular and the ventromedian nuclei and by a posterior

TABLE 1. Summary of the damage sustained in experiment 1 by various parts of the hypothalamus.

Ferret no.	Optic chiasma	Anterior com-missure	Supra-chiasmatic nucleus		Supra-optic nucleus		'Median eminence nucleus'	Fornix		Paraventricular nucleus		Ventro-medial nucleus	
241	+ +	.	+ +	+ +	.		+ +		+ +	+	+ +	+	+ +
263	+ +	.	+ + +		.		+ +	+ + +		.	.	.	.
287	+ +	.	+ + +	+ + +	+		+ +		+ + +	+	+	+ +	+
290	+ + +	.	+ + +	+ + +	+ +		+ +	+		+	+	+	+ +
299	+ +	.	+ + +	+ + +	.		+ +	+ + +		+ +	+ +	+	+
292	+ +	.	+ +	+ +	.		+ + +	+ + +	+ + +	+ + +	+ + +	+	+ +
313	+ + +	.	+ + +	+ + +	+ + +	+ + +	+		+ +	.	.	.	.
317	.	.	+	+	.		+	+ + +	+ + +	+ + +	+ + +	+ +	+
314	+ +	.	+ + +	+ + +	+ + +		+ +	+ + +	+ + +	+ + +	+ + +		+ + +
301	+ +	+	+ + +	+ + +	+ + +		.	+ + +		+		.	
311	+ + +	.	+ + +	+ + +		+ +	.	.		.		.	
309	+ + +	.	+ + +	+ + +	+ +	+ +	.	.		.		.	
288	+ +	.	+ + +	+ + +	.		+	+ + +		+ + +	+		+
295	+	.	+	+ +	.		+	+ + +	+ + +	+ + +	+ + +	.	
306	+ + +	+	+ + +	+ + +	+ + +	+ + +	.	+ + +	+ + +	.		.	

+ + + indicates complete elimination or severe damage, + + indicates moderate but well marked damage and + indicates the occurrence of some destruction. Each part of some bilateral structures has been listed separately. The animals are arranged in the order in which they came into oestrus.

extension of the nucleus supra-opticus ('median eminence nucleus'). The latter structure consists of a basal layer of cells in the floor of the hypothalamus which are prominent between the posterior border of the optic chiasma and the pituitary stalk.

The pituitary stalk remained undamaged in all ferrets except number 263, in which part of the stalk had been damaged by the lesion. No damage could be found in the hypothalamus of any of the blank-operated ferrets. In some ferrets the optic chiasma was damaged to sufficiently large an extent to make it seem likely that the animals were blind. Special tests of vision were not used.

Body and organ weights. Body weights at the time of death and the weights of the endocrine glands of the ferrets are presented in Table 2, where the operated and blank-operated animals are subdivided according to the activity of the gonads at death. None of the animals had been pregnant. When the organ weights of oestrous operated and blank-operated ferrets are compared there is little difference between the ovarian and uterine weights. The slightly larger adrenal glands and smaller thyroid glands in the operated ferrets are not significantly different from the controls.

The body weights of the ferrets anoestrous at death are lower than of those killed during oestrus. This may be accounted for by the fact that these animals had been oestrous for considerable periods and it has been noted on a number of occasions (Donovan & van der Werff ten Bosch, unpublished) that ferrets show a gradual loss of weight during oestrus. In no case was there any indication of the occurrence of post-operative hyperphagia.

Ovulation. Two operated oestrous females (241, 263) were mated 4 weeks after the onset of oestrus and killed a few days later. Luteinized follicles were observed in the ovaries of 263. In addition, corpora lutea were found in the ovaries of 299 and 301 although these females were kept in individual cages isolated from the male. Ferret 299 had then been oestrous for 10 weeks and ferret 301 for 6 weeks.

Thyroid function. The results obtained from measurement of the 4 hr uptake of ^{131}I are presented in Table 3. In the blank-operated oestrous group the figures appear to be fairly uniform and range between 12·4 and 24·8%. There is considerable scatter in the group of oestrous operated animals, with the uptakes in six of the ten ferrets being 10·3% or less. Examination of the anatomical findings in the brains of the operated animals (Table 1) fails to show any correlation between damage to any particular hypothalamic structure listed and a low ^{131}I uptake.

The occurrence of a high or low uptake of radioactive iodine could not be associated with an advancement of the onset of oestrus. Histological changes in the thyroid glands could not be linked with high or low levels of ^{131}I uptake.

Experiment 2. During the winter of 1956–57 twenty-three ferrets were

TABLE 2. Body and organ weights of the ferrets in lesion experiment 1

Ovarian activity at death	Operated						Blank-operated					
	Ferret no.	Body wt. (g)	Adrenals (mg)	Thyroid (mg)	Ovaries (mg)	Uterus (mg)	Ferret no.	Body wt. (g)	Adrenals (mg)	Thyroid (mg)	Ovaries (mg)	Uterus (mg)
Oestrous	241	590	103	37·5	144	1123	293	950	72	86	177	510
	263	650	132	42	205*	1037†	296	720	64	70	91	1206
	290	650	138	38	71	813	297	650	93	39·5	98	1136
	299	800	111	86	109*	1312†	300	560	82	61	93	1295
	314	650	129	33·5	86	1269	302	610	64	48·5	87	1375
							307	790	114	49	109	1325
	301	590	67	46	123*	4912†	310	580	114	48	92	1215
	309	570	112	50	67	971	315	700	111	49	98	1146
	311	600	98	55·5	69	730	316	500	94	30·5	58	828
							318	710	103	54·5	114	879
	288	600	120	19	99	914	Av.	677	91	54	102	1091
	295	660	95	51	104	1589						
	306	560	108	38·5	109	1060						
	Av.	629	110	45	108	1058						
Anoestrous	287	430	75	22·5	71	435	305	470	82	41	69	254
	292	470	91	59	127	328	312	410	106	39·5	76	279
	313	410	65	34·5	85	233						
	317	430	92	30·5	96	230						

* Contained luteal tissue † Omitted from average.

studied. Electrolytic lesions were placed in the anterior hypothalamus of six, in the posterior hypothalamus in four, in the thalamus in two, and in the amygdaloid region in seven animals. Blank operations were carried out on four animals (insertion of the electrode into the anterior hypothalamus in two and into the posterior hypothalamus in two). The operations were carried out during the middle of November, that is some 6 weeks before the date of those of the previous year, and the results are set out in Text-fig. 1*D*, *E*, *F*, *G*.

TABLE 3. Four-hour uptake of ^{131}I by the thyroid gland expressed as the percentage of the dose given (experiment 1)

	Operated		Blank-operated	
Ovarian activity at death	Ferret no.	Thyroid ^{131}I (% dose given)	Ferret no.	Thyroid ^{131}I (% dose given)
Oestrous	241	2·8	296	16·8
	263	0·7	297	20·6
	288	10·3	300	22·3
	295	7·6	302	12·4
	299	6·1	307	20·6
	301	14·1	310	18·7
	306	26·1	315	15·4
	309	26·2	316	17·3
	311	22·8	318	24·8
	314	9·4	—	—
Anoestrous	292	8·0	305	13·5
	313	10·4	312	12·3
	317	16·4	—	—

Onset of oestrus. Lesions of the amygdaloid region of the brain, or blank operations, failed to advance the onset of oestrus. The eleven animals in these two groups (*F* and *G*) became oestrous at the normal time of year. Lesions in the posterior hypothalamus and in the thalamus also failed to hasten oestrus, and in these animals the occurrence of vulval swelling was spread over 7 weeks. Four of the six animals with lesions in the anterior hypothalamus became oestrous before any of the animals with lesions elsewhere in the brain. Two showed vulval changes in December, 4 and 6 weeks post-operatively and 9 weeks before any of the control animals. The onset of oestrus was again markedly advanced in ferrets with anterior hypothalamic lesions.

Position of lesions: anterior hypothalamus. The lesions were very similar to those of Expt. 1 although the optic chiasma escaped damage in ferrets 329, 332 and 336. Generally the lesion extended posteriorly from the chiasma into the anterior hypothalamus but did not reach the pituitary stalk; except in 329 where it extended between the damaged fornices back over the median eminence without harming the latter structure. In ferrets 331 and 332 one limb of the fornix was destroyed by a unilateral extension of the lesion. The damage to the hypothalamus was very small in 336 and consisted of a narrow tubular lesion which passed from the thalamus through the paraventricular nucleus to the floor of the hypothalamus on one side of the brain.

Posterior hypothalamus. The lesions lay in the region of the mammillary body in this group of animals. Thus in ferrets 330 and 337 one mammillary body was destroyed and the other damaged and in each a cylindrical lesion extended upwards through the thalamus to the habenular complex unilaterally. The lesion in ferret 339 was rather similar but confined to one mammillary body. A mid-line lesion was present in 340, placed basally between the mammillary bodies, and destroyed the medial halves of each structure.

Thalamus. Two animals sustained marked thalamic damage. The bulk of the medial thalamus above the mammillary bodies was destroyed in ferret 343 and one habenular complex was atrophic, whilst in 344 the lesion began below the habenular nuclei and extended posteriorly in the mid-line. Thalamic damage was also noted in ferrets 330, 336 and 337 and consisted of extensions of a hypothalamic lesion along the electrode track.

Amygdaloid area. The lesions in this series of six animals consisted of destruction of the surface of the temporal lobe lateral to the optic chiasma.

Fertility. With the exception of ferrets 320 and 322, all the animals were mated and ovulation occurred in all except number 328, as evidenced by subsequent pregnancy or pseudopregnancy. Ferret 328 was with a male for 4 weeks but the vulval swelling did not subside. At the end of this period she was found dead and post-mortem examination showed the presence of a kidney abscess and widespread peritonitis. Litters were produced and successfully reared by ferrets 323, 325, 326, 327, 331, 332, 333, 334, 335, 336, 337, 339, 341, 342 and 343.

Body and organ weights. The body weights of the animals of the different groups showed no marked differences. Hyperphagia was not apparent in any of the ferrets. The weights of the adrenal and thyroid glands and of the ovaries are not recorded in detail because some animals within each group were oestrous, whereas others were anoestrous at the time of death. There were no gross deviations from normal in any one animal.

Stimulation of the hypothalamus

During the season 1954–55 electrical stimulation of the hypothalamus was carried out in nine animals of which only three proved of value. Six animals had to be discarded because of failure of the electrode or connexions, or because they were stimulated for very short periods. Ferret 92 was stimulated for 0·5–4 hr a day by the remote control technique from 16 October to 29 October, continuously in the light-tight box at 0·5 V during the period 10 November to 9 December, and subsequently for 16 hr a day until 15 December. No sign of onset of oestrus was detected. The tip of the unipolar electrode was embedded in the optic chiasma of ferret 92. Another animal, ferret 78, was subjected to stimulation for approximately 16 hr daily at 0·5 V in darkness for a month from 15 December to 15 January. This ferret remained anoestrous until killed on 25 January. The position of the electrode in this case was marked by a lesion in the posterior hypothalamus, medially in the mammillary body. The longest period of stimulation was that applied to ferret 76, which sustained electrical excitation of the hypothalamus at 0·6 V in the light-tight box from 22 November to 9 December and then for about 16 hr daily until 4 February. An induction coil was then substituted for the alternating current and stimulation continued until 17 March. The ferret then became ill with a head abscess and died on 13 April. Subsequent study of the brain revealed gross destruction of a large area of the medial hypothalamus and thalamus. The ferret failed to become oestrous.

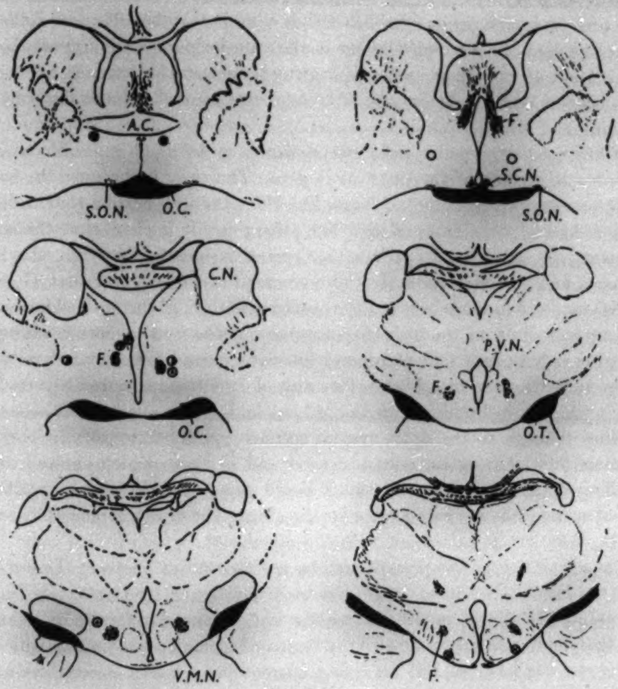
TABLE 4. Details of the stimulation applied to the hypothalamus of seven ferrets in 1955/56

Ferret no.	Period of stimulation (days)	Intensity (V)	Killed
181	36 (9–21 Dec.; 30 Dec.–23 Jan.)	0·8	8 Mar.
202	64 (12 Dec.–14 Feb.)	0·6	8 Mar.
238	32 (4 Nov.–6 Dec.)	0·5–1·0	9 Dec.
249	16 (2 Nov. –18 Nov.)	0·5–1·0	9 Dec.
250	26 (2 Nov.–28 Nov.)	0·5–1·0	28 Nov.
253	66 (4 Nov.–9 Jan.)	0·5–1·0	8 Mar.
282	61 (15 Dec.–14 Feb.)	0·8	8 Mar.

The 1955–56 series of experiments was more successful in that seven ferrets were studied with periods of stimulation of the hypothalamus of from 16 to 66 days. In order to stimulate a larger mass of hypothalamic tissue bipolar electrodes were used but in no animal was oestrus advanced. Details of the periods of stimulation and the voltages used will be found in Table 4; the position of the electrode tips is plotted in Text-fig. 2. Stimulation of the hypothalamus at intensities below 1 V did not evoke any somatic reactions. However, higher voltages produced clear behavioural changes such as alerting, sniffing and a rage reaction often accompanied by discharge of the scent glands in the region of the anus. In ferret 238 a lesion had been produced in the course of stimulation which extended transversely above the optic chiasma into the pre-optic area.

The longest periods of stimulation were those applied to ferrets 202, 253 and 282 where hypothalamic excitation continued over 2 months or more. In ferret 202 the electrodes were positioned on each side of the pituitary stalk lateral to the ventromedial nuclei. The tips were basal and reached the floor of the brain. In ferret 253 one electrode tip was situated lateral to the fornix at a point lateral to the paraventricular and ventromedial nuclei, whilst the other was located in a similar position but anterior to the paraventricular nucleus. The tips of the electrodes were very close together in ferret 282, being about 250 μ apart, and lay between the columns of the fornix a little behind the posterior border of the optic chiasma.

Ferret 181 was stimulated for 36 days and here again the electrodes spanned the columns of the fornix dorsal to the optic chiasma. Ferret 250 was subjected to hypothalamic stimulation for 26 days through electrodes which ended anterior and ventral to the anterior commissure, and in ferret 249 the electrodes lay in the region of the medial forebrain bundle on each side between the anterior commissure and optic chiasma. This animal was studied over a period of 16 days.



Text-fig. 2. To illustrate the sites of electrical stimulation. The positions of the tips of the electrodes are indicated by circles in this series of transverse sections through the diencephalon of the ferret; ◑ = 181, ⊗ = 202, ○ = 249, ● = 250, ⊙ = 253, ⊕ = 282.
A.C., anterior commissure; *C.N.*, caudate nucleus; *F.*, fornix; *O.C.*, optic chiasma; *O.T.*, optic tract; *P.V.N.*, paraventricular nucleus; *S.C.N.*, suprachiasmatic nucleus; *S.O.N.*, supra-optic nucleus; *V.M.N.*, ventromedial nucleus.

Histological study of the ovaries, uterus and thyroid glands of the stimulated ferrets did not reveal the presence of any abnormal feature.

DISCUSSION

Under natural conditions of light the female ferret kept in a laboratory displays vulval swelling at the beginning of March, and only occasionally does oestrus occur earlier in the year (Donovan & van der Werff ten Bosch, 1956*c*). The present investigation establishes that oestrus may be advanced following the placement of electrolytic lesions in the anterior part of the hypothalamus. Fifteen out of twenty-one animals bearing lesions in the anterior hypothalamus became oestrous before any of twenty-nine control animals did so at the beginning of March.

It has not been possible to locate particular areas which must be damaged in order to accelerate the onset of oestrus. This may be because the lesions in the present series were too large, but the possibility exists that a diffuse area of the hypothalamus is involved. However, it is clear that the region damaged by effective hypothalamic lesions is located between the optic chiasma and pituitary stalk. In this connexion it is of interest that Thomson (1954) made an incision between the posterior margin of the optic chiasma and the anterior limit of the hypothalamus, or cut the optic chiasma sagittally, in seven ferrets kept under ordinary laboratory conditions, and that two of these animals came into heat before any of twenty-four normal controls; a result which is in keeping with the present finding.

Since damage to the optic system seemed equally extensive in operated animals in which oestrus was advanced and in those which showed vulval swelling at the normal time of year it would appear that interference with the visual system was not responsible for the effect. The observations of Thomson (1951, 1954) are in agreement with this conclusion.

The specificity of the present results, as reported in abstract (Donovan & van der Werff ten Bosch, 1956*b*) has been questioned by Herbert & Zuckerman (1957, 1958) who report that oestrus was advanced in ferrets by lesions in the thalamus or adjacent areas of the brain or by blank operations in which the electrode was inserted but no lysing current passed. These conclusions are contrary to our results. In the present study sixteen ferrets were subjected to blank operations without advancement of the onset of heat; they came into oestrus in March. Further, the four blank-operated animals of the 1956–57 series (Expt. 2) became oestrous at the same time as the twelve blank-operated animals of 1955–56 (Expt. 1) despite the fact that they were operated upon 9 weeks earlier. Also, in seven animals with lesions in the amygdaloid region vulval swelling was observed at the usual time, as was the case in six ferrets with lesions in the posterior hypothalamus and thalamus. There is thus little evidence to favour the view that 'non-specific' operations on the brain of ferrets promote the secretion of gonadotrophin and hasten oestrus. No explanation can be advanced for the conflict between the results of Herbert & Zuckerman (1957, 1958) and those of the present work.

The occurrence of ovulation in one of the two animals mated in the first experiment and the high incidence of pregnancy in experiment 2 indicate that the release of ovulating hormone following coitus is not disturbed. Presumably the neural mechanisms in the hypothalamus activated by the stimulus must be situated outside the area of the lesion. In two females ovulation occurred despite isolation from the male. This finding is somewhat surprising since the ferret normally ovulates only after coitus (Marshall, 1904) but might indicate the occurrence of an over-secretion of pituitary gonadotrophin. Marshall (1940) has described the presence of luteal tissue in the ovaries of a ferret submitted to irradiation with ultra-violet light.

The studies carried out on the effect of stimulation of the hypothalamus on the onset of oestrus are not extensive and the results cannot be regarded as conclusive. It is difficult to maintain intact leads and to continue stimulation in ferrets for very long periods. Nevertheless, in view of the fact that in several animals stimulation was continued for at least 2 months without any sign of follicular activation the findings would seem to be of value. Upon comparison with the position of the lesions inducing oestrus it will be seen that stimulation was applied to this hypothalamic area in ferrets 249, 181 and 282 but the electrodes were a little anterior in ferret 250, posterior in 202 and were obliquely positioned in the region in ferret 253.

The results just presented make it clear that destruction of part of the anterior hypothalamus in the ferret may be followed by advancement of the onset of oestrus. Several explanations may be put forward. It may be postulated that the discharge of gonadotrophic hormone from the hypophysis follows absorption of break-down products of nervous tissue resulting from electrolysis. These could act directly on the hypophysis or elsewhere. Alternatively, it may be suggested that an irritative effect of the lesion exerted upon the surviving nerve cells on its borders may be responsible for activation of the hypophysis. Two points argue against this view. The first is that it has not so far been possible to induce oestrus in the ferret by electrical stimulation of the hypothalamus. Whilst a negative result on this point cannot be conclusive in view of the nature of the evidence and the small number of experiments carried out, it would appear that if an irritant effect was responsible for the result produced by the lesion, then it should be somewhat easier than is actually the case to elicit oestrus by electical stimulation of the hypothalamus. Secondly, the oestrus evoked by the lesion appears to be normal in character. Such ferrets may remain in oestrus, like normal unmated animals, for many weeks—an occurrence that would not be expected to follow irritation of damaged nerve cells or absorption of break-down products.

It is considered possible that lesions in the anterior hypothalamus produce oestrus by destruction of or interference with an area which normally inhibits the secretion of pituitary gonadotrophin, thus permitting the discharge of pituitary hormone. Under conditions of sexual quiescence the area would exert a tonic restraining effect on gonadotrophin secretion. Stimuli known to cause pituitary activation, such as exposure to prolonged illumination, would then act by depressing the anterior hypothalamus with consequent discharge of gonadotrophin in increased amount. The experiments involving stimulation of the hypothalamus would seem to be in full accord with this concept, in that electrical excitation of the hypothalamus would be expected to delay the onset of oestrus and not to advance it. A further test of this hypothesis would be to continue stimulation into spring and early summer in order to see whether oestrus is deferred.

The findings of precocious puberty in children with pathological lesions in the hypothalamus have been explained in comparable terms (Weinberger & Grant, 1941). Experimental lesions in the anterior part of the hypothalamus of the infantile rat have been found to hasten the time of vaginal opening, whereas lesions in the pre-optic region were ineffective (Donovan & van der Werff ten Bosch, 1959). The latter findings have been interpreted to indicate the participation of an inhibitory hypothalamic mechanism in the processes underlying sexual maturation. It is of interest that several immature ferrets were used in the current work and it may be said that in these (263, 287, 290) puberty was advanced.

Several groups of workers have found that electrolytic lesions placed above and anterior to the median eminence of the tuber cinereum depress thyroid function. Greer (1951, 1952) recorded thyroid atrophy in the rat with lesions in this area and this observation was confirmed by Bogdanove & Halmi (1953). In the dog, Ganong *et al.* (1955) found a definite depression of thyroidal ^{131}I uptake and histological evidence of atrophy of the thyroid gland in animals with lesions in and just above the anterior end of the median eminence (see also Greer, 1957). In the ferret the lesions which markedly depress the uptake of ^{131}I have been found to lie rather basally in the anterior hypothalamus, whilst the lesions often also involved the paraventricular and ventromedial nuclei. The results in the ferret would therefore be in accord with those of workers employing other species with regard to functional changes in the thyroid gland. Since the histological appearance of the thyroid varies from one part to another in the gland of the ferret it is felt that little reliance can be placed on morphological criteria, and the decrease in thyroid weight, shown in Table 2, was doubtful.

SUMMARY

1. The effects on the reproductive cycle of the ferret of electrolytic lesions placed in the anterior hypothalamus and other parts of the brain have been examined.

2. Lesions were made in the anterior hypothalamus during the winter anoestrous period in twenty-one ferrets. In the majority of these the onset of oestrus was markedly hastened; ten animals came into heat within 4 weeks of operation. Sixteen blank-operated control animals were also studied, but in none was oestrus advanced.

3. Control lesions in the posterior hypothalamus (four ferrets), thalamus (two), and amygdaloid region (seven) failed to hasten the onset of heat.

4. Thyroid function was also studied in the ferrets with anterior hypothalamic lesions and their blank-operated controls by an ^{131}I uptake technique, and it was found that basal lesions in the anterior hypothalamus markedly depressed the uptake of the tracer.

5. The effect of electrical stimulation of the hypothalamus was studied in sixteen animals, three of which were subjected to continuous excitation for 2 months or more. There was no indication of any release of gonadotrophin by the stimulus.

6. It is suggested that lesions in the anterior hypothalamus of the ferret advance oestrus by destruction of or interference with an area which normally inhibits the secretion of gonadotrophin and so permits the discharge of pituitary hormone.

We wish to express our appreciation of the advice and encouragement so readily given by Professor G. W. Harris, F.R.S. Our thanks are extended to Messrs W. F. Piper and P. G. Kirkby for their invaluable technical assistance, and to Mr F. Smith for photographic aid.

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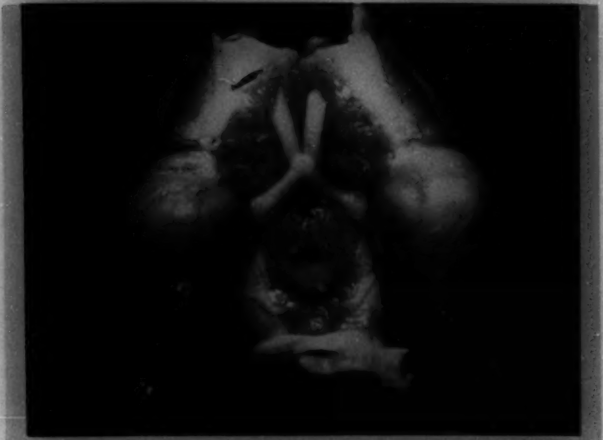
EXPLANATION OF PLATE

PLATE 1

Fig. 1. View of ventral surface of brain of ferret 314. Note the dark damaged area posterior to the optic chiasma and extending forward over the left optic tract.

Fig. 2. Transverse section through the anterior hypothalamus of ferret 314, showing the region in the mid line destroyed by the lesion. (Loyez stain; 25μ).

1



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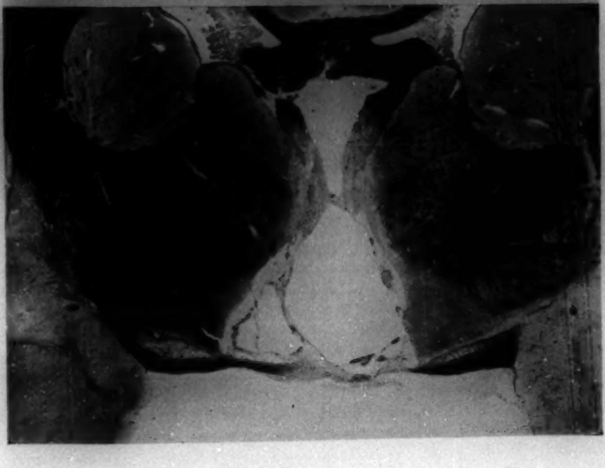

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EFFECT OF SENSITIZATION ON THE ELECTROLYTE COMPOSITION OF CERTAIN TISSUES IN THE GUINEA-PIG

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Electrolyte changes after an antigen injection were reported by Schittenhelm, Erhardt & Warnat (1928), who studied the effects of a sensitizing dose on the potassium and calcium in the blood and certain tissues of the rabbit and dog. These authors also determined the values of these cations after a second dose of antigen. Fox, Nelson & Freeman (1951) and Einbinder, Nelson & Fox (1954) described the changes in sodium and potassium which were observed in mice after the second dose of the antigen.

This paper is designed to examine the changes in water content and cation distribution in tissues produced by a sensitizing dose of antigen, since these effects have thus far received little attention. In order to find out if these changes are specific for the condition of sensitization, control experiments were carried out in which the blood volume was reduced by haemorrhage, or the water and sodium contents were lowered by the injection of hypertonic glucose solution.

METHODS

Female guinea-pigs were sensitized by a single subcutaneous injection of 0·75 ml. of 5% crystalline egg albumin (Parke, Davis and Co.) in distilled water, or by a single intraperitoneal injection of 0·5 ml. horse serum. The animals were killed at the height of sensitivity 21–24 days after the sensitizing dose. Sensitivity was tested by challenging a piece of terminal ileum with the antigen in the isolated organ bath.

In order to determine how far simple withdrawal of blood might produce results comparable with those seen in the sensitized animal, blood was withdrawn from the heart by direct cardiac puncture. Assuming a value for blood of 8% of body weight, about one-fifth of the blood volume was removed from each animal. The animals were killed 2 hr after the withdrawal of blood.

Water and sodium depletion was carried out by the method described by Yannet & Darrow (1940). The guinea-pigs were given 5% glucose 10 ml./100 g body weight by intraperitoneal injection, and were killed 3 hr afterwards.

All animals were killed after weighing, by bleeding from the jugular vein. Tissue wet and dry weights were obtained and the difference between them was considered to be the water content of the tissue. The tissues were dissolved in fuming nitric acid in a boiling water-bath, and analysed with a flame photometer, using appropriate filters and standard solutions for sodium and potassium content.

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The hair on the left buttock was cut very short and a specimen of skin was excised and cleaned free from underlying tissue; it was then blotted with Green's filter paper to remove superficial blood. Skeletal muscle was removed from the outer side of the left thigh, only muscle belly being taken for examination. A portion of terminal ileum was taken and the mesenteric border was cleaned of fat and blood vessels. The lumen was exposed and wiped clean before the piece was placed in the specimen tube. The apex of the heart was amputated so that parts of both ventricles were included in the specimen, from which pericardium and endocardial clots had been removed. Lung tissue was obtained from the periphery of the lung. The trachea was cut above the carina and the tissues at the root of the lung were cleaned of lung tissue, fat and glands; the sample obtained in this way was considered to be composed mainly of bronchial tree.

RESULTS

Sensitization with egg albumin

The mean values with standard errors for the water, sodium and potassium contents of tissues from twenty normal guinea-pigs and from fourteen sensitized with egg albumin are shown in Table 1. A statistically significant reduction in each of these constituents was found in the skin, a decrease in the water and sodium in the bronchial tissue, and of sodium and potassium in the ileum of the sensitized animals. The reference tissues, cardiac muscle and lung, derived from the sensitized animals, did not exhibit such changes nor were the findings in skeletal muscle different in the sensitized animals from those in the normal guinea-pigs.

TABLE 1. Tissue composition of normal (*N*) and egg albumin sensitized (*S*) guinea-pigs: mean ± S.E.

		Water	Sodium	Potassium
		(%)	(m-equiv/100 g dry tissue)	
Skin	*N*	66·4 (±1·2)	23·3 (±0·9)	9·6 (±0·5)
	S	61·3 (±0·7)	19·8 (±1·2)	7·3 (±0·4)
Skeletal muscle	*N*	76·8 (±0·2)	11·5 (±0·6)	50·4 (±1·1)
	S	76·7 (±0·2)	11·1 (±0·6)	51·3 (±0·7)
Ileum	*N*	79·5 (±0·5)	31·5 (±0·8)	46·3 (±0·9)
	S	79·1 (±0·6)	26·4 (±1·0)	40·5 (±2·2)
Cardiac muscle	*N*	78·5 (±0·5)	21·7 (±0·6)	41·7 (±0·6)
	S	79·6 (±0·3)	22·0 (±0·4)	42·1 (±0·9)
Lung	*N*	78·2 (±0·2)	32·6 (±0·9)	37·8 (±0·6)
	S	77·8 (±0·5)	35·1 (±1·6)	41·7 (±1·4)
Bronchial tissue	*N*	71·3 (±1·5)	31·2 (±1·3)	18·8 (±1·5)
	S	65·9 (±1·7)	25·9 (±1·3)	17·2 (±1·2)

These comparisons between groups were extended to the study of individual animals from which skin was removed before and after sensitization. Two sets of guinea-pigs, each composed of five animals, were submitted to skin biopsy, and one set was then sensitized. After 21 days a further specimen was taken from each guinea-pig.

The untreated group had a mean water loss of 0·4 % in the skin. The sodium was reduced by 1·4 m-equiv/100 g dry tissue, and the mean potassium level fell by 1·6 m-equiv/100 g dry tissue. The sensitized animals showed a reduction in each of these components, the mean water level being decreased by 1·8%, the mean sodium value by 3·4 m-equiv/100 g dry tissue, and the mean potassium content by 3·0 m-equiv/100 g dry tissue. Each of the observations on the sensitized animals was lower than the initial figure, while two of the controls produced raised values. The findings on the individual animals thus provide confirmatory evidence of the group observations.

Some animals which received egg albumin did not give a positive response when the terminal ileum was treated with the antigen in the organ bath. The values obtained from these guinea-pigs did not differ from the normal, and the deviations from normal which the sensitized animals presented were not found. The mean figures for ten such non-sensitized animals are shown in Table 2. The fact that the results from the non-sensitized animals were similar to the normal values suggests that the changes noted in the sensitized guinea-pigs were not merely due to the egg albumin injected, but were the result of the process of sensitization.

TABLE 2. Tissue composition after egg albumin injection in the absence and presence of sensitization: mean ± s.e.

		Water (%)	Sodium	Potassium
			(m-equiv/100 g dry tissue)	
Skin	*NS*	67·9 (±0·6)	23·5 (±1·0)	9·7 (±0·4)
	S	61·3	19·8	7·3
Ileum	*NS*	80·5 (±1·7)	28·9 (±2·0)	43·9 (±4·8)
	S	79·1	26·4	40·5
Bronchus	*NS*	74·9 (±1·4)	33·1 (±0·8)	22·1 (±0·4)
	S	65·9	25·9	17·2

S, sensitized; *NS*, not sensitized.

Sensitization with horse serum

The work of Parratt & West (1957) suggested that egg albumin liberated 5-hydroxytryptamine in the rat after a second or anaphylactic dose. The effect was thus an increase in capillary permeability. For this reason, it seemed desirable to compare the effects of another antigen.

The findings in eight animals after sensitization with horse serum are shown in Table 3. The results were similar to those in guinea-pigs sensitized with egg albumin. The water, sodium and potassium contents in the skin were decreased, though only the last was highly significant. The findings for ileum were significant reductions in each component, and in the bronchial tissue the lowered sodium value was highly significant, the water and potassium changes being smaller. The raised lung water content was an isolated finding which does not have any apparent explanation at this time.

Effect of haemorrhage

The mean values for the tissue composition of five guinea-pigs after haemorrhage are shown in Table 4. The water changes were small in the tissues examined, and some sodium reduction occurred in the skin, ileum and bronchial tissues. The unusual finding was a rise in water and sodium in skeletal muscle. This is in agreement with J. Chaudhury's observations (personal communication) on cats and may be explained on the basis of adrenalin dilatation of the muscle blood vessels. A. F. Munro (personal communication) estimated the catecholamines in the cat and found that approximately 1–2 hr after a severe haemorrhage the blood level, particularly of the adrenalin, was raised.

TABLE 3. Tissue composition of normal (*N*) and horse serum sensitized (*S*) guinea-pigs: mean ± S.E.

		Water (%)	Sodium	Potassium
			(m-equiv/100 g dry tissue)	
Skin	*N*	66·4	23·3	9·6
	S	64·0 (±1·1)	21·5 (±1·0)	6·6 (±0·5)
Skeletal muscle	*N*	76·8	11·5	50·4
	S	77·3 (±0·4)	12·2 (±0·6)	52·2 (±1·3)
Ileum	*N*	79·5	31·5	46·3
	S	75·1 (±0·9)	24·8 (±2·6)	32·1 (±2·4)
Cardiac muscle	*N*	78·5	21·7	41·7
	S	79·6 (±0·3)	23·3 (±0·7)	42·6 (±0·7)
Lung	*N*	78·2	32·6	37·8
	S	79·0 (±0·3)	33·2 (±1·7)	39·1 (±0·8)
Bronchial tissue	*N*	71·3	31·2	18·8
	S	70·2 (±1·1)	26·9 (±1·2)	15·2 (±1·6)

TABLE 4. The effects of haemorrhage on the water, sodium and potassium content of tissues of the normal guinea-pig

	Water (%)	Sodium	Potassium
		(m-equiv/100 g dry weight)	
Skin	66·5	21·4	8·1
% change	—	−8·1	−16·2
Skeletal muscle	78·9	12·4	51·4
% change	+2·3	+7·8	+2·0
Ileum	80·7	26·8	40·5
% change	+1·5	−14·9	−12·5
Cardiac muscle	80·0	20·5	41·2
% change	+1·9	−5·5	−1·2
Lung	78·1	30·9	39·3
% change	—	−5·2	+4·0
Bronchial tissue	72·2	27·6	20·4
% change	+1·3	−11·5	+8·5

Effect of water and salt depletion

The injection of hypertonic glucose results in the removal of water from the extracellular fluid space, and this is accompanied by sodium withdrawal. There is rapid compensation by the tissues, producing alterations in their water and sodium contents. These effects were compared with those which followed the process of sensitization.

The mean values for the observations on fifteen guinea-pigs are shown in Table 5. There were highly significant reductions in the water, sodium and potassium contents of the skin and bronchial tissue, and of the water and sodium in the skeletal muscle and ileum. The results for cardiac muscle and lung did not present any change from the normal values.

Comparison of these findings with the observations on sensitized animals showed that the changes in skin, ileum and bronchial tissues were of the same order. However, the reduction in the sodium content of skeletal muscle following the injection of glucose did not occur with sensitization.

TABLE 5. Tissue composition of normal guinea-pigs depleted with intraperitoneal glucose; mean ± S.E.

	Water (%)	Sodium	Potassium
		(m-equiv/100 g dry weight)	
Skin	60·4	19·0	7·3
% change	−9·0	−18·5	−24·0
Skeletal muscle	75·3	9·8	50·0
% change	−2·0	−15·0	−0·8
Ileum	75·6	26·7	41·8
% change	−5·0	−15·3	−10·0
Cardiac muscle	80·9	22·0	42·4
% change	+3·0	+1·3	+1·7
Lung	78·2	31·4	38·9
% change	—	−3·7	+2·9
Bronchial tissue	63·2	23·0	13·9
% change	−11·4	−26·3	−26·1

DISCUSSION

Fox *et al.* (1951) had already demonstrated that in anaphylactic shock the plasma sodium fell and they concluded that there was a movement of sodium into the tissues. Examination of the tissues in the supersensitive state did not reveal any change in the sodium content of the uterus, ileum or skeletal muscle (Hughes & McDowall, 1958), but loading with sodium or treatment with DOCA led to a smaller retention of sodium, and depletion experiments using glucose showed a greater effect in the sensitized animal than the normal. Hughes and McDowall therefore concluded that the tissues of sensitized animals were excessively permeable to sodium.

Our experiments showed that the skin, bronchial tissue and ileum were affected by the sensitization process. This was not the result of egg albumin injection, since it did not occur if the guinea-pig was not sensitized, and there were similar findings after horse serum sensitization. Neither reduction in the vascular space nor of the extracellular water and sodium produced a similar series of events, and the deduction is therefore drawn that this effect is specific. No evidence is offered at this stage for the nature of this change.

SUMMARY

1. Female guinea-pigs were sensitized with egg albumin or horse serum. Analyses of skin, skeletal muscle, ileum, cardiac muscle, lung and bronchial tissue were carried out.

2. The water and sodium contents of the skin, ileum and bronchial tissue were reduced by sensitizing the guinea-pigs.

3. Skin biopsy before and after sensitization with egg albumin on the same guinea-pig confirmed the observations made on the group of animals as an effect on the individual.

4. In the absence of sensitization egg albumin did not produce these alterations in tissue composition.

5. Reduction in blood volume by haemorrhage, and lowering of the water and sodium of the body by the injection of hypertonic glucose did not produce the effects which characterized sensitization.

This work formed part of a thesis of one of us (R. St J. B.) for the degree of Ph.D. of the University of London. A grant towards the cost was received from the Asthma Research Council.

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THE EFFECT OF ALTERATIONS OF PLASMA SODIUM ON THE SODIUM AND POTASSIUM CONTENT OF BONE IN THE RAT

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(*Received* 17 *December* 1958)

Within recent years attention has been focused on the possible role of bone in relation to plasma sodium regulation. It has been shown, for example, that relative to other tissues, the Na content of bone is high. It has also been suggested that bone sodium is labile and may act as a freely available reservoir for the preservation of plasma sodium level (Woodbury, 1956). Loeb (1933) found that, after adrenalectomy, dogs lost sodium in their urine. Flanagan, Davis & Overman (1950) concluded that the fall in serum sodium which occurred at the same time could not be completely explained on the basis of sodium loss in the urine. They postulated that in adrenalectomized dogs, some sodium entered tissue cells, and they considered bone as one of the tissues into which sodium must have entered. In contrast, however, Stern, Cole, Bass & Overman (1951) found no definite change in the sodium content of bone in adrenalectomized dogs.

Past work thus gives no very positive indication as to the functions of bone as a sodium reservoir. The purpose of the present work was, therefore, to reinvestigate the function of bone in this respect and, in particular, to determine the importance of the adrenal gland and adrenal cortical secretions in relation to bone sodium.

METHODS

The first requirement in the present investigation was the accurate determination of the extracellular space of bone. Previous workers (Woodbury, 1956) used the chloride space method for this determination. Nevertheless, most workers agree that elsewhere in the body, apart from radioisotope methods, inulin and sucrose space determinations are the most accurate of the techniques available (Ross & Mokotoff, 1951). Accordingly the attempt was made in the present work to adapt the inulin space technique to the present problem.

Apart from the intracellular water of the osteocytes, most of the water in bone is in the Haversian system and connecting canaliculi. Bone matrix also contains a small fraction of the total bone water. The desiccation method measures the total water content of bone. All methods for the determination of extracellular space, however, give only the volume of the Haversian system. The difference between the total water and inulin space, therefore, represents water in the bone matrix as well as intracellular water. This amount of water will subsequently be referred to simply as matrix water, since cells form only a very small part of the total matrix volume.

In the present work acute alterations in plasma sodium were produced by water loading in the conscious rat. The adrenal glands were removed in one series of experiments and in another series, cortisone was given intraperitoneally after adrenalectomy. The effects of these three procedures on bone water, sodium and potassium were studied.

Operative procedure. Wistar strain albino rats of 200 g body weight were lightly anaesthetized with ether. Both renal pedicles were ligated through a dorsal incision and an inulin solution (22% w/v) was injected into an external jugular vein in the dose of 0·5 ml./100 g body weight (Ledingham, 1953). The animals were deprived of food and water, and after 5 hr heparin (Liquemin, Roche, 500 u./kg body weight) was administered intraperitoneally. After 6 hr blood was collected from a carotid artery. Bone specimens from the four limbs were pooled after removal of the epiphyses. The bones were crushed with bone forceps and the marrow was carefully wiped off with Na-free filter paper. The pieces of compact bone were then placed in tubes of known weight for the determinations described below.

Bone inulin space. No reference could be found to previous estimations of bone inulin. It was necessary to find some method of bringing the bone into solution without hydrolysing the inulin content to the enol stage, which would diminish the colour developed with the addition of resorcinol-thiourea to the bone solution. It was also necessary to determine whether bone contained any inuloid material which would in itself increase the colour density of the bone solution. It was further necessary to decide on the optimum period of equilibration of plasma inulin with bone.

The method finally adopted for dissolving bone was to leave the weighed pieces of bone during the night in stoppered tubes containing 2·0 ml. of 30% HCl at room temperature of 23° C. The bone was completely dissolved: 4·5 ml. of 4 N-NaOH was added to neutralize the solution, which was made up to 10 ml. with distilled water and filtered (Whatman 42). 3·0 ml. of the filtrate was used to develop the colour reaction as described by Ross & Mokotoff (1951). The results of the preliminary investigation will be described later; they were, however, such as to justify the method, to show that the use of bone 'blanks' was not necessary and to demonstrate that a 3 hr equilibration period was sufficient.

Adrenalectomy. Bilateral adrenalectomy was carried out through a dorsal incision under light ether anaesthesia and the animals were kept for 3 days on standard cube diet and a drinking solution of NaCl 0·5% (w/v) (Dosekun & Mendel, 1958).

Intraperitoneal injection of cortisone following adrenalectomy. Immediately after the removal of the adrenal glands of the rats, cortisone acetate B.P. (Cortisyl; *Roussel*) was injected intraperitoneally in a dose of 1 mg/100 g body wt, and subsequent daily injections of the same dose were given; the last dose was given 2 hr before the animal was killed on the morning of the fourth day after adrenalectomy. The rats were kept on normal cube diet and given tap water to drink.

Bone water. 200–400 mg wet bone was weighed to within 0·1 mg, dried during the night at 100° C and then re-weighed after cooling to constant weight. The total water content was calculated from the weight loss, in terms of ml./kg wet wt. of bone.

Plasma inulin was determined by the method of Roe, Epstein & Goldstein (1949). The inulin concentration of extracellular space was taken as 1·1 × plasma inulin (Ledingham, 1954). Extracellular water was calculated from the bone and plasma inulin and expressed in ml./kg wet wt. of bone.

Bone Na and K. As Ca increases the flame photometer reading of Na, it was necessary to precipitate this along with the organic material of bone before the determination of bone Na. Bone specimens were heated for 15 min with 1·0 ml. 50% HNO_3 till dissolved and the solution was appropriately diluted with ion-free distilled water. The bone Ca was precipitated by the method of Bergstrom & Wallace (1954), using appropriate modifications in reagent volumes. The precipitate after centrifuging was re-washed four times until all the Na and K content was removed and the washings were pooled for Na and K estimation on the flame photometer. Results are expressed in m-equiv/kg wet wt. of bone. As osteocytes form only a small part of the total matrix bulk, it is practically impossible to determine separately the quantity of intracellular Na and K; the difference between the total and extracellular ions will therefore be subsequently referred to as matrix Na and K.

Plasma Na and K were measured by flame photometry after suitable dilution. The concentration of Na in the extracellular fluid is taken as 0·96 × plasma Na to allow for the Gibbs–Donnan effect.

Water loading. Distilled water 12·5 ml./100 g body wt. was injected intraperitoneally after light ether anaesthesia and ligation of both renal pedicles; an hour after this another dose of distilled water (12·5 ml./100 g body wt.) was given. The animals were killed 3 hr after the second administration of water. This method caused a significant lowering of plasma Na, as will be seen from the results described below.

RESULTS

Validity of techniques

Measurement of bone inulin. In nine experiments a known amount of inulin (2·0 ml. of 0·1 mg/ml.) was added to 0·2–0·4 g wet weight of rat bone. The bone pieces were weighed to within 0·1 mg in stoppered tubes. 2·0 ml. of 30 % HCl was added to this, and the mixture left during the night at room temperature. The bone was completely dissolved; the inulin was recovered as described in Methods. That a colour was developed in these experiments showed that the chemicals used in the above concentrations and quantities

TABLE 1

A. Inulin recovery after dissolving bone with HCl and adding inulin 2·0 ml. × 0·1 mg/ml.; figures expressed in mg inulin

Rat no.	Amount of inulin added	Amount of inulin recovered
1	0·20	0·19
2	0·20	0·20
3	0·20	0·20
4	0·20	0·20
5	0·20	0·18
6	0·20	0·20
7	0·20	0·20
8	0·20	0·19
9	0·20	0·20
Mean	0·20	0·19 ± 0·05 S.E.

$P < 0·5$

B. Inulin space determinations 3 and 6 hr after giving 0·1 mg inulin; Figures expressed as ml./kg wet wt. of bone

Group *A*		Group *B*	
Rat no.	3 hr after inulin	Rat no.	6 hr after inulin
1	62·0	1	92·0
2	76·0	2	67·0
3	99·0	3	63·5
4	82·5	4	87·0
5	89·0	5	99·0
6	76·1	6	84·0
Mean	80·8		82·1
S.E.	±4·1		±5·3

$P < 0·2$

did not completely hydrolyse the inulin added. The amount of inulin recovered as shown in Table 1*A* was 0·19 mg ± 0·05 which was not significantly different from the amount of 0·20 mg inulin added to the bone specimens at the beginning of the experiments. This also proved that bone contained no inuloid material, and bone 'blank' experiments were therefore omitted. The above method having proved satisfactory was used in the subsequent experiments.

Equilibration of plasma inulin with bone. As it was necessary to determine the optimum time required for inulin to come into equilibrium between plasma and bone, a preliminary investigation was carried out on six rats in each of two groups, in which bone was collected for inulin estimation 3 and 6 hr respectively after intravenous administration of inulin 1·0 mg/ml. The bone inulin was derived by dividing the bone inulin by plasma inulin, the results being expressed as ml. inulin space/kg wet wt. of bone. The results obtained from these experiments are shown in Table 1*B*. The mean inulin space of bone 3 hr after giving 0·1 mg of inulin intravenously to six rats was 80·8 ± 4·1 and the inulin space 6 hr after giving the same amount of inulin intravenously to six other rats was 82·1 ± 5·3. These results are not significantly different ($P < 0{\cdot}2$). They therefore show that a period of 3 hr is enough for equilibration of bone and plasma inulin.

Inulin space and matrix water in bone of control rats

The mean values (± S.E.) obtained in six control rats for total water, inulin space and matrix water of bone were 141·3 ± 6·8, 81·1 ± 5·7, and 60·2 ± 4·03 ml./kg wet wt., respectively.

The effect of water loading on bone water and electrolytes

Dosekun & Mendel (1958) have shown that the presence of a large volume of intraperitoneal fluid did not affect the measurement of inulin space in tissues. The effect of water loading on bone water and electrolytes was studied in six experiments. Rats were lightly anaesthetized with ether, both renal pedicles were ligated and inulin was given intravenously. After 3 hr equilibration, water was injected intraperitoneally in two equal doses of 12·5 ml./100 g body wt. at 1 hr interval. After a further 3 hr of water loading, blood was collected from a carotid artery and bone specimens were removed for analysis. The results given below are compared with six controls in which bone was collected for analysis 6 hr after giving inulin intravenously but without water loading. The absorption of water from the peritoneal cavity was satisfactory, the residual water not being greater than 2·5 ml./100 g body wt in any of the experiments.

Plasma Na and K. The mean plasma Na in the control group was 138 ± 1·6 and that in the water-loaded group 110·6 ± 1·1 m-equiv/l.; this result represents a highly significant lowering of plasma Na ($p < 0{\cdot}001$). The mean plasma K in the control group was 5·4 ± 0·1 and that in the water-loaded group was 4·6 ± 0·5 m-equiv/l. This is not a significantly lower value than the control value ($P < 0{\cdot}2$). It can therefore be seen that water loading led to a highly significant lowering of plasma Na but not of plasma K.

Bone water. The results obtained for the total water and inulin space in the water-loaded and control groups are shown in Table 2. It can be seen that after water loading for 3 hr, although the increase in total water was probably significant, there was no significant change in the inulin space. Since the inulin space is a measure of the volume of the extracellular space, the small change in total water must therefore be due to slight entrance of water into osteocytes and bone matrix.

TABLE 2. The effect of water loading on the total water and inulin space of bones in the rat; figures expressed in ml./kg wet wt. of bone

	Total water	Inulin space
Control rats	141·3 ± 6·8	81·1 ± 5·7
Water-loaded rats	161·3 ± 6·3	85·9 ± 5·7
P	< 0·02	< 0·6

TABLE 3. The effect of water loading on the total Na, extracellular Na, matrix Na and total K of rat bone; figures expressed as m-equiv/kg wet wt. of bone

	Total Na	Extracellular Na	Matrix Na	Total K
Control rats	199·1 ± 4·2	10·7 ± 2·4	188·4 ± 4·5	22·4 ± 1·5
Water-loaded rats	189·3 ± 4·5	9·1 ± 2·02	180·2 ± 5·6	19·1 ± 1·2
P	< 0·1	< 0·5	< 0·2	< 0·1

Bone Na. The results are shown in Table 3. It can be seen that water loading caused no significant change either in the total Na content of bone or in the extracellular Na and therefore no change in the matrix Na which is obtained by deducting the extracellular from the total Na.

Bone K. As can be seen in Table 3, water loading has caused no significant change in the mean total K content of bone as compared with the mean value obtained from six control rats. The extracellular K fraction is less than 1% of the total bone K and matrix K may therefore be regarded as similar to total K. The extracellular K has therefore not been shown in the Table.

The role of the adrenal glands and cortisone on bone water and electrolytes

The results described below were obtained from experiments on six rats kept on normal saline for 3 days after adrenalectomy. After this period, blood and bone were removed from the rats for analysis. A second group of six rats were given cortisone for 3 days after adrenalectomy and then killed and blood and bone examined. These results are tabulated in Tables 3 and 4.

Plasma Na and K. As is shown in Table 5, adrenalectomy has lowered the plasma Na significantly whereas cortisone was able to maintain plasma Na at practically normal level after adrenalectomy. As regards K, it will be seen from the same table that adrenalectomy caused a significant rise in plasma K, whereas cortisone maintained the plasma K at a normal level after adrenalectomy.

TABLE 4. The effect of adrenalectomy and cortisone following adrenalectomy on the total water and inulin space of rat bone; figures expressed as ml./kg wet wt. of rat bone

	Total water	Inulin space
Normal (control rats)	141·3±6·8	81·1± 5·7
Adrenalectomized rats	139·3±5·2	80·5±6·0
P	< 0·9	< 0·6
Adrenalectomized rats given cortisone	171·6±5·4	138·6±11·5
P	< 0·001	<0·001

TABLE 5. The effect of adrenalectomy and cortisone following adrenalectomy on the plasma Na and K of the rat; results expressed as m-equiv/l.

	Plasma Na	Plasma K
Normal	138·8±1·6	5·4±0·1
Adrenalectomized rats	119·5±1·0	6·3±0·5
P	< 0·01	< 0·01
Adrenalectomized rats given cortisone	135·6±1·5	5·2±1·0
P	< 0·6	< 0·5

TABLE 6. The effect of adrenalectomy and cortisone after adrenalectomy on the total Na, extracellular Na, matrix Na and total K of rat bone; figures expressed as m-equiv./kg wet wt. of bone.

	Total Na	Extracellular Na	Matrix Na	Total K
Normal	199·1± 4·2	10·7±2·4	188·4± 4·5	22·4±1·5
Adrenalectomized rats	167·3± 3·0	9·3±0·17	157·9±3·2	5·9±0·7
P	< 0·001	< 0·5	< 0·01	< 0·001
Adrenalectomized rats given cortisone	210·4±20·8	18·2±2·4	192·2±20·3	12·3±1·48
P	< 0·7	< 0·001	< 0·5	< 0·001

Bone water. From Table 4 it can be seen that adrenalectomy caused no significant change in total water nor did it cause any significant change in the inulin space. In a group of rats, however, to which cortisone was administered for a period of 3 days after adrenalectomy, different results were obtained. Thus there was a highly significant rise in total water and there was also a significant rise in inulin space.

Bone Na. From Table 6 it can be seen that adrenalectomy caused a highly significant fall in the mean total value of bone Na but no significant change in the extracellular Na; the resultant significant decrease in the matrix Na is therefore due to the loss of Na from bone matrix. In the same table it is clear that cortisone given for 3 days after adrenalectomy prevented the loss of Na from bone, since there is no significant difference between the mean total Na in the control rats and in those receiving cortisone after adrenalectomy. The extracellular Na has, however, been highly significantly increased by cortisone.

Bone K. It is clear from Table 6 that adrenalectomy caused a highly significant loss of K from bone. After the administration of cortisone for 3 days there was a considerable increase in bone K; the level was, however, still significantly below normal. It should be noted that the total amount of K in bone is small compared with Na.

DISCUSSION

The present work shows that the inulin space method yields an extracellular space volume which is intermediate between that of cardiac and skeletal muscle (Dosekun & Mendel, 1958) and definitely lower than the chloride space of bone as reported by Woodbury (1956). Also acute hyponatraemia did not expand bone inulin space significantly. Woodbury (1956) obtained a similar result from his chloride space determinations in bone.

Woodbury (1953, 1956) and Bergstrom & Wallace (1954) reported that in acute hyponatraemia there was a significant loss of Na from the bone of rats. Woodbury therefore suggested that the released Na probably served, in part, to maintain extracellular Na concentration of plasma and tissues at a level somewhat higher than would obtain if no Na were mobilized from bone. Munro, Satoskar & Wilson (1957), on the other hand, showed that chronic Na deficiency produced no net loss of sodium from rat bone. The present work shows that the total sodium of intact rat bone was not significantly reduced by acute water loading. The present results are therefore in agreement with the findings of Munro *et al.* (1957). As a labile reservoir of Na, bone is unlikely to play a significant part, since its Na appears to be securely bound to its matrix.

That the adrenal glands are critically related to bone Na is clearly shown by the present experiments. Thus adrenalectomy produced a significant loss of Na from bone matrix. Stern *et al.* (1951) did not find a definite loss of sodium in the bone of adrenalectomized dogs, whereas Flanagan *et al.* (1950) postulated from the results of their experiments that adrenalectomy caused absorption of Na by bone. The present results did not accord with the findings of the above workers. When adrenalectomized rats were given an adequate dose of cortisone for 3 days after adrenalectomy, it was found that Na loss from bone matrix was prevented. The increase in total bone water caused by this procedure appears to be due chiefly to an expansion of the extracellular volume of the Haversian system resulting from water retention in this space. There remains a further increase in total bone water which cannot be fully accounted for on the present evidence. Recently, Munro, Satoskar & Wilson (1958), using radioactive technique, have reported findings which are in agreement with the results of the present investigation in respect of Na loss from bone after adrenalectomy and the preservation of bone Na by cortisone following the removal of the adrenal gland.

The mechanism of the adrenal control of bone Na is as yet unknown. It may well be that adrenalectomy, by causing a generalized break-down of bone matrix in the rat, would lead to a passive loss of Na bound to the matrix. On the other hand, the adrenal cortex may play a specific humoral role in effecting a net concentration of Na in bone and in the maintenance of bone Na in a stable condition.

As regards K the present work indicates that adrenalectomy caused a significant loss of K from bone which cortisone was unable to prevent completely. Although the quantity of bone K is very small compared with bone Na, cortisone does not appear to be adequately effective in the preservation of bone K after adrenalectomy. It is possible that some other adrenocortical hormone is needed either alone or in addition to cortisone in order that bone K shall be maintained at a normal level.

SUMMARY

1. The inulin space of bone has been determined and the effects on the Na and K content of bone of alteration in plasma Na, adrenalectomy and the administration of cortisone after adrenalectomy have been studied.

2. With lowering of plasma Na by water loading there was no significant loss of Na or K from bone.

3. Adrenalectomy produced significant diminution in bone Na and K but no change in inulin space.

4. Cortisone administration was able to maintain the normal level of bone Na but not of bone K after adrenalectomy. It also caused a considerable expansion of the inulin space of bone.

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THE EFFECT OF LOWERING THE AMBIENT TEMPERATURE ON THE METABOLISM OF THE NEW-BORN PIG

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Part of the chemical regulation of body temperature is the rise in oxygen consumption which follows the exposure of animals to low ambient temperatures. It has been extensively investigated in adults and there would appear to be a muscular element in it due to an increase of tone and to shivering, and also a non-muscular element (Burton & Edholm, 1955). Giaja (1925) studied new-born animals and showed that their increase in O_2 consumption was relatively small and varied much from species to species: in rabbits the response increased greatly in the first few days of life. The rat has probably got no control over its body temperature at birth and makes no response on that day (Adolph, 1951, 1957), but it can be shown to raise its O_2 consumption slightly by the fifth day of life if suitable ambient temperatures and lengths of exposure are selected for the tests (Hahn & Koldovský, 1958). These experiments have all been of short duration. As new-born animals go, observation in the field suggests that the new-born pig has probably got fairly good control of its body temperature, and although Holub, Forman & Ježková (1957) found that its O_2 uptake did not rise when it was exposed to a temperature of 3° C for 30 min, Mount (1958) has shown that this was probably a mistake; for in his, also short-term, experiments new-born piglets, whether starving or fed, have always responded to a fall in the ambient temperature by a rise in their O_2 consumption.

In spite of the results of these short-term experiments, practical experience has proved that new-born piglets do not survive more than 30 hr in a cool environment unless they are fed, and the cause of death is thought to be hypoglycaemia (Morrill, 1952; Morrill & Sampson, 1952; Goodwin, 1957*a*, *b*). The calf and foal survive much longer. These effects of temperature are matters of medical interest, for the new-born infant will survive many days without food in a warm environment but appears to die of hypoglycaemia if exposed to cold (Mann & Elliott, 1957).

Little is known about the effect of the ambient temperature on the non-gaseous metabolism in men or animals. Lusk (1928), in reviewing the earlier work of Rubner and others, concluded that the excretion of N was not affected by the exposure of adult dogs or human beings to cold, and this is what might have been expected from what is known of the muscular response, but You, You & Sellers (1950) showed that if the temperature in which adult rats were being kept was reduced from about 25 to about 1·5° C, and if at the same time their food intake was kept the same, their excretion of nitrogen almost doubled itself in the next days. No one else appears to have studied this. The effects of thyroidectomy and adrenalectomy on both the O_2 consumption and the excretion of N have, however, been studied and are complicated (You *et al.* 1950; Hahn & Koldovský, 1958).

The present experiments were designed to find out more about the general metabolic response of the new-born piglet to a low ambient temperature, with the ultimate object of bringing the result to bear upon the treatment of premature and new-born infants which have got profoundly chilled.

METHODS

The experiments have been made on twenty-two new-born piglets, taken 6–8 at a time from each of three litters. The sows were the result of an original Large White × Essex cross subsequently mated, as a rule, with pure-bred Large White boars. The piglets were removed from the sow as soon as they were born and placed in a room at about 25° C and weighed when they were dry. A little blood was taken from the cord, or the sow, or both, for the determination of the concentration of urea in it at birth. The piglets were then taken to the laboratory in a warm box in a heated car. This move took about 30 min in all. The weights were checked on another balance and the animals were then paired by weight. One of each pair was placed in a metabolism cage in a room at 31° C and the other in a similar cage at 12° C. The animals were given no food or water and their rectal temperatures were taken at once, after about 8 hr, and then more frequently. All their urine was collected under toluene. At the end of about 24 hr the animals were removed in turn from the cages and placed on plastic sheeting; blood was removed from the heart and they were then killed by injecting air into it. If they had not recently done so the animals usually emptied their bladders at this moment, and if they did the urine was removed from the plastic sheeting and added to the volume of urine which had already been passed. The animals were weighed as quickly as possible after they were dead and allowance was made for the blood which had been removed. They were then opened at once and duplicate portions of liver, muscle and heart removed for glycogen determination. Any urine found in the bladder was also kept for analysis. The bodies were placed on tared trays, weighed, and dried to constant weight. The composition of the bodies of new-born piglets has been taken from the analyses of litter mates, killed at birth, and the figures agreed closely with those previously obtained in this department (Spray & Widdowson, 1951). The results differed little from one animal to another, for new-born piglets of average weight have a very uniform chemical structure.

The cages and chemical methods employed have been those described by McCance & Widdowson (1956). Fat, which was not then being estimated, was determined by breaking up the dried carcasses and extracting the product with petroleum ether. Glycogen was determined by the method of Good, Kramer & Somogyi (1933). The small sample of blood required for the determination of blood sugar was received into a tube containing a few crystals of an oxalate-fluoride mixture to prevent clotting and glycolysis. The remaining blood was prevented from clotting by the addition of heparin or was allowed to clot and the serum separated as soon as possible. The method used for measuring the blood sugar was that of Nelson (1944). Fructose was separately determined on some samples, and was found to have a concentration of about 2 mg/100 ml. in the serum 24 hr after birth (Goodwin, 1957*b*).

RESULTS

Piglets kept at 31° C maintained their rectal temperatures quite near the adult level for the whole 24 hr (Fig. 1). This result was in keeping with many observations made in this department over the last 4 or 5 years; at temperatures of about 25° C piglets, even without food or water, have never displayed any distress for at least 40–48 hr. Figure 1 also shows that animals kept at 12° C maintained their rectal temperature quite well for some 8–12 hr, but that the temperature then began to fall and fell progressively faster. The experiments were terminated when the rectal temperature of the animals had fallen to between 20 and 23° C.

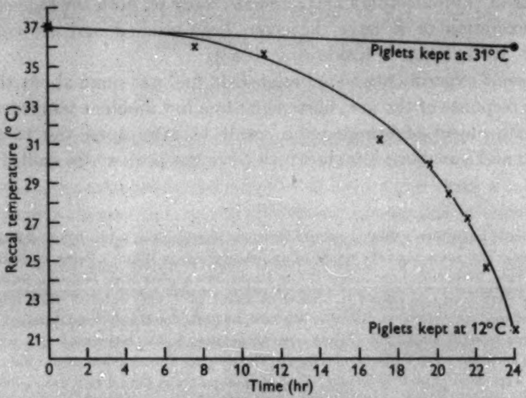

Fig. 1. Average rectal temperatures of the piglets in one litter, four kept at 12° C and four kept at 31° C for 24 hr. Piglets from other litters gave very similar results, but the times at which the temperatures were taken were not necessarily the same.

When their temperatures began to fall the animals kept at 12° C moved about less freely than the others and spent most of the time sitting on their haunches or lying in the cage. Fluid collected in the subcutaneous tissues, and the dependent parts were marked by the pattern of the floor of the cage on which the animals were lying. The hairs stood on end and the coats felt very cold to the touch, especially towards the end, although the animals had a pink bloom all over and did not appear cyanosed as one might have expected. Babies *in extremis* from cold have been said to have a similar pink flush (Mann & Elliott, 1957). These particular piglets never had convulsions, but convulsions have been seen subsequently in cold piglets which were being used for another experiment.

Table 1 shows the change in the animals' body weights, the amount of water excreted by the kidney, the serum urea at birth and death, the total nitrogen excreted in the urine and the tissue protein catabolized. In this and subsequent tables the figures for the animals in each of the three litters have been averaged and given separately, and standard deviations of the averages for the eleven piglets at each of the two environmental temperatures have been calculated. All the animals lost weight and the ambient temperature did not make very much difference to the amount. The urine volumes were larger in the cold, but since none of the animals was taking liquid by mouth, these volumes, as in infants of this age (McCance & Widdowson, 1954), were probably fixed by the osmotic load to be excreted. The serum urea was considerably higher at death in animals kept at 12° C, and the excretion of urea and other nitrogenous end products nearly twice as great. This was probably the main reason for the larger volume of urine. The last two columns of Table 1 show that in the animals kept in the colder environment the catabolism of tissue protein was more than three times as great as it was in the animals kept at 31° C. This effect of cold, therefore, hitherto reported only in adult rats (You *et al.* 1950), applies to another species and operates at a very early age. It is probably a general phenomenon.

Table 2 shows the effect of the ambient temperature on the excretion of sodium, chloride, potassium and phosphorus in the urine and the concentrations found in the serum at death. The ambient temperature made little difference to the amount of sodium excreted, but piglets at this age never excrete more than trifling amounts even when the serum values are high, and this is why it is so easy in piglets and new-born babies to produce hypertonic expansion of the extracellular fluids (McCance & Widdowson, 1957). More potassium was excreted when the ambient temperature was low, and additional chloride accompanied it (McCance & Widdowson, 1958). The excretion of phosphorus too was higher in the cold. The animals kept at 12° C had lower concentrations of sodium and chloride in their serum at death than the animals kept at 31° C, and higher concentrations of potassium and phosphorus. Since the animals passed no faeces, or only negligible amounts, the movements of sodium and potassium must have taken place within the closed system represented by the body and the urine, and it may be concluded that the sodium and chloride which left the extracellular fluids at the low ambient temperature probably entered the body cells, and the additional potassium found in the extracellular fluids and urine must have left them.

Table 3 shows the N/K and the N/P ratios in the urine and also the ratios of N/K and N/P appearing as end products during the survival of these animals at 12 and 31° C. Considerable use has been made of such ratios in the

TABLE 1. The effect of the ambient temperature on the body weight, the volume of urine, the serum urea, the excretion of total nitrogen and the catabolism of tissue protein

	Loss of body weight (g/kg/24 hr)		Urine vol. (ml./kg/24 hr)		Serum urea (mg/100 ml.)			Total N excretion (mg/kg/24 hr)		N appearing as end products of protein catabolism (mg/kg/24 hr)	
						Death					
Litter	Cold (12° C)	Hot (31° C)	Cold	Hot	Birth	Cold	Hot	Cold	Hot	Cold	Hot
1	109	92	63	42	25	53·9	15·4	194	93	284	63
2	90	67	42	33	21·4	44·3	18·0	184	110	267	90
3	99	107	51	26	21·6	48·6	16·8	154	104	259	85
Average all piglets	98	89	50	34	22·7	48·2	16·7	173	102	267	80
S.D.	±25·2	±21·8	±16·2	±12·7	±2·5	±10·1	±3·2	±72·1	±15·8	±110	±20·3

* N appearing as end products of protein catabolism = N excreted in urine ± the change in the total amount of urea N in the body.

TABLE 2. The effect of the ambient temperature on the excretion of sodium, potassium, chloride and phosphorus and on their concentrations in the serum at death

	Excretion (m-equiv/kg/24 hr)								Serum concn. (m-equiv/l.)							
	Na		K		Cl		P		Na		K		Cl		P*	
Litter	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot
1	0·20	1·15	5·95	2·76	3·57	2·07	—	—	138	147	7·1	4·7	98	117	—	—
2	0·93	0·85	5·35	2·84	3·57	2·74	32·7	2·5	139	144	7·2	5·2	100	110	8·1	3·9
3	0·65	0·22	4·82	3·23	2·98	1·55	14·1	3·4	143	149	7·0	5·0	104	111	—	—
Average all piglets	0·66	0·74	5·35	2·94	3·42	2·12	22·1	2·8	140	147	7·1	5·0	101	113	8·1	3·9
S.D.	±0·59	±0·90	±1·06	±0·46	±1·03	±0·90	±12·7	±2·8	±3·6	±2·6	±0·63	±0·31	±2·9	±3·6	—	—

* m-equiv/100 ml.

urine as indicating the source and nature of materials being catabolized and set free during starvation (Lusk, 1928; Gamble, Ross & Tisdall, 1923; Smith, Yudkin, Young, Minkowski & Cushman, 1949). The subject was discussed by McCance & Widdowson (1954), as it applied to human infants.

If the ratios of N mg/K m-equiv in the muscles of the new-born pig be taken as 214 and of N mg/P mg as 8·75 (Dickerson & Widdowson, 1959), then it is clear that the N/K ratios in the urine at both temperatures were lower than the N/K ratio in the muscle, and so apparently were the ratios of the two elements catabolized. This is not true to the same extent of the N/P ratios, particularly of the ratio of N/P excreted at 31° C. A comparison of the results for the N/P ratios excreted and catabolized shows the importance of not assuming that the two are even approximately equal. They may under special conditions be equal, as in starving adults and older children, but evidently not under the conditions of these experiments. Lowering the ambient temperature may have led to relatively more P than N being catabolized, but the differences were not large. The main conclusion to be drawn from these ratios must be that cold intensified the rate of metabolism but made no great difference to the pattern of the materials being metabolized.

Table 3. The ratios of nitrogen to potassium and phosphorus excreted and catabolized

	In urine				Catabolized			
	N (mg)/ K (m-equiv)		N (mg)/ P (mg)		N (mg)/ K (m-equiv)		N (mg)/ P (mg)	
	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot
Litter 1	32·6	33·5	—	—	40·0	18·5	—	—
Litter 2	34·5	38·8	5·6	44·0	42·3	30·0	5·2	9·5
Litter 3	32·0	32·3	10·9	30·6	52·5	28·0	—	—
Average all piglets	32·5	34·8	7·8	36·5	45·3	25·2	5·2	9·5

N catabolized = N excreted in urine ± the change in the total amount of urea N in the body; K and P catabolized = amount excreted in the urine ± the change in the amount in the extracellular fluids.

Table 4. The effect of the ambient temperature on the concentration of sugar in the blood at death and on the percentages of glycogen found in the liver, muscles and heart

	Blood sugar (mg/100 ml.)		Liver glycogen (g/100 g)		Muscle glycogen (g/100 g)		Heart glycogen (g/100 g)	
Litter	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot
1	9	72	1·04	4·50	0·38	7·85	0·08	0·33
2	28	111	1·89	6·55	0·30	6·10	0·14	0·88
3	9	96	0·36	4·10	0·40	2·60	0·14	0·57
Average all piglets	14	93	1·02	5·12	0·38	5·20	0·13	0·63
S.D.	±15·4	±27·1	±1·11	±1·49	±0·27	±2·42	±0·10	±0·52

Table 4 shows the results bearing directly on carbohydrate metabolism. Animals kept at the higher temperatures maintained their blood sugars within normal limits, but in the colder environment the blood sugars were sometimes extremely low when the animals were killed. The quantities of glycogen found in the muscles and liver were variable, but the results indicate that the low ambient temperature reduced the amount of glycogen in the tissues; but not to vanishing point. The low blood sugars in animals kept at 12° C cannot therefore be attributed solely, if indeed to any extent, to the total exhaustion of all the glycogen stores, but the low body temperatures may have prevented the glycogen being broken down. Hahn (1956*b*) did not study the effect of cold on the levels of blood sugar, but he showed that the exposure of young rats to 10° C, and a fall in their body temperatures to 15° C, reduced the liver and muscle glycogen, though not until the animals were about 18 days old.

TABLE 5. The effect of the ambient temperature on the percentage of water and of fat found in the carcasses at death

	Percentage of water in carcasses			Percentage of fat in carcasses		
Litter	At birth	After 24 hr at 12° C	After 24 hr at 31° C	At birth	After 24 hr at 12° C	After 24 hr at 31° C
1	81·5	83·2	81·3	1·10	0·84	0·86
2	81·3	82·6	81·5	0·99	0·60	0·71
3	80·8	83·2	80·9	1·01	0·55	0·85
Average all piglets	81·2	83·0	81·2	1·03	0·63	0·79
S.D.	±0·67	±0·76	±0·47	±0·08	±0·16	±0·16

Table 5 shows the amount of water and of fat found in the bodies of normal new-born piglets and in those of animals kept for 24 hr at 12° C or at 31° C. The bodies of the animals kept at 12° C contained more water. To some extent this may have been due to a smaller invisible loss of water, but in the main it was due to the increased metabolism of the solids of the body: see also Table 6. Part of these solids was the body fat and Table 5 shows that the bodies of animals kept at 12° C did in fact contain a smaller percentage of fat. The amount of fat, however, in new-born piglets is always very small and the percentages found in these experiments were in agreement with previous findings in this laboratory (Widdowson, 1950). Consequently, whatever the ambient temperature, the fat metabolized by these animals made but a small contribution to their metabolic requirements and accounted for only about a quarter of the solids which disappeared. Table 6 shows this by giving the information available from these experiments as to the amount and nature of the solids metabolized by starving piglets in the first 24 hr of their lives, and the effect of the ambient temperature upon them. Over all the experiments, 17 g of solids was metabolized per kilogram in 24 hr at 31° C. More proteins were broken down in the cold and protein accounted for a higher percentage of the solids which disappeared, but they never accounted for more than a small percentage of the total solids metabolized (Nikitin, 1948; McCance & Strangeways, 1954). The same was true of fat, and fat actually formed a smaller proportion of the total solids metabolized in the cold than in the warm ambient temperature. The bulk of the solids which disappeared at both temperatures was neither protein nor fat and must by inference have been carbodydrate. This accounted for 83–84 % of the solids whether the ambient temperature was low or high. This percentage seems large, but if the skeletal muscles be reckoned to form 29 % of the body weight of a new-born piglet, the liver 2·4 % and the heart 0·8 %, and if the whole organs are assumed to have contained the same percentages of glycogen as those found in the parts taken for analysis (7·2 % in muscle, from new-born piglets, 8·5 % in liver and 1·5 % in heart), the glycogen which disappeared between birth and death was 69 and 67 % of the total solids metabolized in the animals kept cold and warm respectively. The actual figures were 24 g/kg in the animals kept cold and 11·2 g/kg in those kept warm. There are undoubtedly depots of glycogen in the body other than skeletal muscle, liver and heart, and there seems no doubt that carbodydrate provided the main source of energy for these starving new-born piglets.

TABLE 6. The effect of the ambient temperature on the metabolism of the solids in the body, and on the contribution of the tissue proteins, fat and glycogen to this metabolism

Solids metabolized (g/kg/24 hr)

	Total		Protein		Fat		'Difference' i.e. glycogen		'Difference' as % of total	
	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot
Litter 1	38	18	1·8	0·4	1·9	1·7	34·3	15·9	90	88
Litter 2	30	15	1·7	0·6	4·3	3·2	24·0	11·2	80	75
Litter 3	37	17	1·6	0·5	4·8	1·8	30·6	14·7	83	93
Average all litters	35	17	1·7	0·5	4·0	2·4	29·3	14·1	84	83

The solids metabolized were calculated from the loss of weight of the experimental animals, their composition at death, and the composition of healthy litter-mates killed at birth.

DISCUSSION

These experiments are far from perfect, and a major point of criticism must be that the animals were not in a steady state throughout the 24 hr of observation. The rate of metabolism of the bodily reserves and tissue protein might have been found to have been even higher at 12° C if the measurements had only been made for the first 12 hr, for after this the body temperatures of these animals began to fall rapidly and this in itself must have lowered the metabolic rate (McCance & Widdowson, 1957, 1958). Goodwin's (1957*b*) work, moreover, suggests that the blood sugars might not have been so low had the experiment been terminated earlier, and there might have been no change in the serum electrolytes after 12 hr (see below). The fall in body temperature during the second 12 hr may have prevented the break-down of the last of the glycogen. Hahn (1956*b*) suggested, in support of this idea, that exposure to cold did not appear to reduce the tissue glycogen of very young rats because their body temperatures and consequently their metabolic rates fell so rapidly that the effect was masked. Within these limitations, however, these experiments do seem to have opened up new aspects of metabolism in the cold and to offer a method by which they can be relatively easily studied.

The catabolism of tissue protein and its relationship to the metabolism of fat and other non-nitrogenous material seem very fundamental. This is something quite distinct from the forced metabolism of shivering, which classical physiology exempts from any catabolism of tissue protein whatsoever. You *et al.* (1950) found that the thyroid and adrenal glands were not essential for the increased excretion of nitrogen in adult rats kept in the cold, although both glands were necessary for survival there. The position in the new-born pig remains to be investigated, but the Prague workers have made a start on the new-born rat (Hahn, Křeček & Křečková, 1956; Hahn, 1956*a*) and found that the thyroid gland does not begin to participate in thermoregulation as measured by oxygen consumption till the animals are at least 14 days old. The effect of a low ambient temperature on protein catabolism will also require study in connexion with the food requirements for growth.

Little seems to be known about the effects of a subnormal body temperature on the metabolism of electrolytes, but Robinson (1950) demonstrated with tissue slices *in vitro* that conditions which interfered with the metabolism and oxygen uptake of the cells—cold among them—prevented the cells maintaining their normal chemical structure. It has now been shown by a study of several muscular and glandular organs that on chilling water enters the cells and with it sodium and chloride. Some potassium is extruded (Hercus, McDowall & Mendel, 1955; Rixon & Stevenson, 1956; Leaf, 1956; Whittam, 1956; Whittam & Davies, 1956; Cort & Kleinzeller, 1958). The findings in the present investigations are in keeping with the early stages of such a process and, if so, they are the effects of a fall in the temperature of the body, not part of the metabolic response to a low ambient temperature.

Since the response of the new-born piglet to a low ambient temperature seems to resemble in so many ways that of the new-born infant, a study is now being made of the resuscitation of animals after they have reached the state described in this investigation.

SUMMARY

1. Normal new-born piglets were kept in metabolism cages without food or water for 24 hr at 12 and at 31° C.

2. The rectal temperatures of the animals kept at 12° C were reasonably well maintained for 8–12 hr, but by 24 hr had fallen to 20–23° C.

3. The ambient temperature made little difference to the loss of weight.

4. Animals kept at 12° C (*a*) had higher serum ureas, excreted more total

N and catabolized more tissue protein than the others, (*b*) had very low blood sugars and less glycogen in their tissues.

5. The carcasses of the animals kept at 12° C contained a higher percentage of water and a lower percentage of fat and other solids than those of animals kept at 31° C.

6. After 24 hr at 12° C the concentrations of sodium and chloride in the serum were lower and those of potassium and phosphorus higher than after 24 hr at 31° C. These differences were due to movements of these electrolytes within the body. More potassium and considerably more phosphorus were excreted at 12 than at 31° C.

7. Carbohydrate provided most of the energy for starving new-born piglets, whether they were kept at 12 or 31° C.

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THE CAUSE OF THE SUPERSENSITIVITY OF SMOOTH MUSCLE TO NORADRENALINE AFTER SYMPATHETIC DEGENERATION

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Meltzer (1904) was the first to describe the supersensitivity of the radial muscle fibres of the iris of the cat's eye to adrenaline, after extirpation of the superior cervical ganglion and degeneration of the post-ganglionic fibres. His observation was soon confirmed by Elliott (1905). In 1931 Burn & Tainter showed that while the denervated iris was supersensitive to adrenaline it was almost completely insensitive to tyramine and ephedrine. In 1949 Burn & Hutcheon showed that the supersensitivity of the denervated iris to adrenaline was not so great as its supersensitivity to noradrenaline. Whereas the supersensitivity was on the average about tenfold for adrenaline, it was about fiftyfold for noradrenaline.

The cause of the supersensitivity has hitherto been unexplained. Recently we observed supersensitivity in different circumstances. In studying the action of the alkaloid reserpine, we found that when it was administered by intraperitoneal injection, the tissues became supersensitive to noradrenaline and adrenaline. We observed this in spiral strips of the thoracic aorta of the rabbit (Burn & Rand 1958*a*) and in the vessels of the dog's hind leg (Burn & Rand, 1958*b*). We observed also that noradrenaline could be extracted from the aorta of the normal rabbit and dog, but that none could be extracted from the vessels of animals treated with reserpine. The results suggested that the disappearance of noradrenaline from the artery walls might be responsible for their supersensitivity. We therefore asked the question whether the supersensitivity which follows degeneration of the sympathetic nerves was also due to a disappearance of noradrenaline from a peripheral store. The answer seemed likely to be that it was, since von Euler & Purkhold (1951) and Goodall (1951) had shown that such a loss occurred in the spleen and other organs.

The present experiments were carried out on the iris and spleen of the cat. We chose the iris because it was the first organ in which supersensitivity was demonstrated, and we chose the spleen because volume changes can be accurately recorded, and the threshold dose of noradrenaline to produce contraction can be determined. A preliminary note of some of our findings has appeared (Burn & Rand, 1958*c*).

METHODS

In treating cats with reserpine we prepared a 1% (w/v) solution using 20% ascorbic acid as solvent. We gave 3 mg/kg by intraperitoneal injection on the first day, and the same dose or sometimes a smaller dose on the second day. The cats were kept in a room at 27° C and used for experiment on the third day.

In making observations on the dilatation of the iris the cat was anaesthetized with urethane and the eyes were equally illuminated with a light just sufficiently bright to keep the pupils constricted to a narrow slit. Injections of sympathomimetic amines were made intravenously into the femoral vein by one of us, while the other watched the eyes. (Where necessary the iris had been denervated on one side by removal of the superior cervical ganglion in an aseptic operation; observations were made 11–17 days later.)

To determine the amount of noradrenaline in the iris the eyeball was removed and washed in saline. The iris was immediately cut out together with its attachment to the sclera, dried between filter paper and put in the deep-freeze for 1 hr or longer. It was cut up with scissors and ground with sand in a mortar (which had been cooled in the deep-freeze) together with 1 ml. acid saline (pH 4). It was transferred to a graduated conical centrifuge tube (10 ml.) and replaced in the deep-freeze for 1 hr or longer. It was thawed and spun at 2500 rev/min for 20 min. The supernatant was poured off and stored in the deep-freeze until the assay was performed. The noradrenaline content was expressed as μg noradrenaline per iris. For the assay, male rats weighing about 200 g were given atropine 1 mg/100 g subcutaneously, and some minutes later were deeply anaesthetized with ether. A tracheal tube was inserted and the rat was then pithed by passing a steel rod through the eye and down the spinal cord. Artificial respiration was given at 80 per minute. A polythene tube (1 mm diameter) was passed into the jugular vein for intravenous injections, and a glass cannula was tied into the carotid artery and connected to a Condon manometer (Shipley & Tilden, 1947; Condon, 1951, 1953; Muscholl & Vogt, 1957). Observations on contractions of the spleen were made in spinal cats, the spleen being drawn through an incision in the body wall and enclosed in a plethysmograph (Burn, 1952). For denervation the spleen was also drawn out of the body wall in an aseptic operation, the splenic artery was exposed and the nerve fibres accompanying the artery were cut; observations on the denervated spleen were made about 15 days later. To determine the noradrenaline content, the whole spleen was washed in saline and dried between filter paper. The connective tissue capsule, fat and external blood vessels were dissected off, and the whole spleen or the middle portion was weighed and put in the deep-freeze for 1 hr or more. It was then treated as described for the iris, using for the extraction 5 ml. saline (pH 4) per gram, the extract being assayed for noradrenaline on the rat blood pressure. Six estimations were carried out in duplicate by von Euler's method (1946); they gave similar results.

RESULTS

Noradrenaline content of iris. We began by making observations on the noradrenaline content of the iris of a series of eight normal cats, and obtained a mean figure of 0·17 μg noradrenaline per iris. We then examined cats in which the iris of the right side was denervated, making the observations in parallel on the normal and denervated organs. The figures are given in Table 1. An example of an assay on the rat blood pressure is shown in Fig. 1. We found that our values for the normal iris of cats in which one iris was denervated were less than half the values we had obtained for the irides from unoperated cats.

However, the values for the denervated organs were very low, and much less than those for the corresponding normal irides. The mean result for the denervated side was one-twelfth of that for the normal side, and was near the limit of what was detectable.

Supersensitivity of reserpine-treated iris. The fourth column of Table 1 gives figures for cats treated with reserpine. The values were also very low. Figures have already been published by Burn & Tainter (1931) and by Burn &

TABLE 1. Noradrenaline content of iris of cat (μg per iris)

	Unoperated cats	Operated cats		Reserpine-treated cats
		Normal	Denervated	
	0·2	0·05	0·003	0·012
	0·1	0·08	0·012	0·007
	0·15	0·035	Nil	0·02
	0·14	0·025	Nil	0·012
	0·16	0·08	0·015	0·006
	0·26	0·125	0·008	0·012
	0·24	0·05	0·005	
	0·08			
Mean	0·17	0·061	0·005	0·011

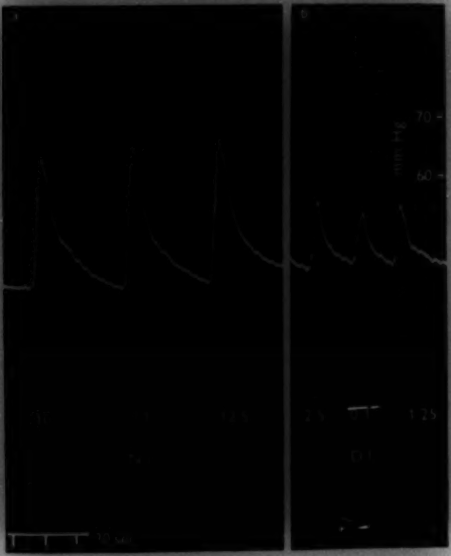

Fig. 1. Rat blood pressure. (*a*) Comparison of extract of normal iris (N.I.) with noradrenaline; 0·1 ml. N.I. is intermediate between 10 ng and 12·5 ng. (*b*) Comparison of extract of denervated iris (D.I.) from same cat with noradrenaline; 0·1 ml. D.I. is less than 1·25 ng.

Hutcheon (1949) for the minimal dilating amounts of noradrenaline, adrenaline and tyramine on the normal and denervated iris when determined by the method we have used. These figures, together with figures for cats treated with reserpine which we have now determined, are given in Table 2. In cats treated with reserpine the iris was found to be supersensitive to both noradrenaline and adrenaline, though the degree of supersensitivity appeared to be rather less than in the denervated irides. This difference was almost certainly due to the difficulty in ensuring that a peripheral tissue such as the iris received the full effect of reserpine.

TABLE 2. Threshold amounts causing dilatation of iris

	Normal iris			Denervated iris			Reserpine-treated		
	Adrenaline (μg)	Noradrenaline (μg)	Tyramine (mg)	Adrenaline (μg)	Noradrenaline (μg)	Tyramine (mg)	Adrenaline (μg)	Noradrenaline (μg)	Tyramine (mg)
	2	—	0·3	0·2	—	2·0	0·2	5·0	> 4
	1	—	0·1	0·1	—	> 8·0	1·0	2·5	> 4
	2	30	—	1·0	1·0	—	0·5	10·0	> 4
	2	25	—	0·1	0·4	—			
	7·5	100	—	0·5	2·0				
Mean	3	52	0·2	0·4	1·1	5·0	0·6	6·0	> 4

TABLE 3. Noradrenaline content of cat spleen (μg/g)

	Normal	Denervated	Reserpine-treated
	1·5	0·24	0·09
	0·75	0·14	0·01
	1·2	0·125	0·2
	1·75	0·075	0·125
	1·0	0·15	0·2
	0·6	0·3	0·18
		0·45	0·062
		0·16	0·012
			0·175
			0·125
Mean	1·13	0·2	0·12

Noradrenaline content of spleen. We knew from the work of von Euler & Purkhold (1951) that denervation reduced the amount of noradrenaline in the spleen of the sheep. We confirmed the finding in the cat, and showed that a similar reduction occurred in the spleen of the cat treated with reserpine. The results are given in Table 3. Denervation reduced the noradrenaline content of the spleen to a mean value of 18 % of the normal; the highest figure in the denervated spleen was lower than the lowest figure in the normal spleen. Treatment with reserpine was more effective than denervation; it reduced the noradrenaline content to 10 % of the normal.

Supersensitivity in the spleen. The smallest amounts of noradrenaline which, when injected intravenously in the spinal cat, caused contraction of the spleen are given in Table 4. The results show that both the denervated spleen

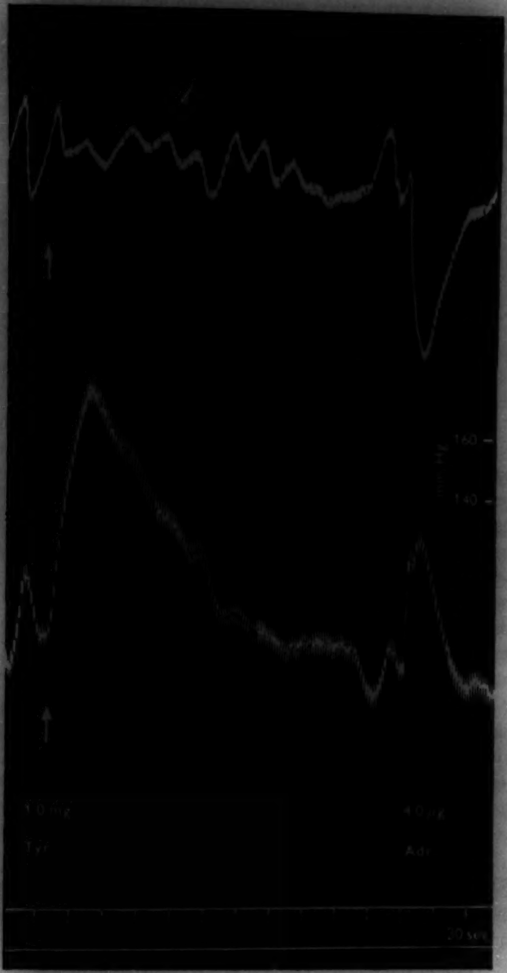

Fig. 2. Spinal cat. Upper record is the volume of the spleen denervated 2 weeks previously. 1·0 mg tyramine caused a rise of blood pressure but did not cause contraction of the spleen. The effect of 4 μg adrenaline is also shown.

and the spleen in a cat treated with reserpine are much more sensitive to noradrenaline, and contract in response to less than one-tenth of the amount required to cause contraction in the normal spleen. Not only so, but amounts of tyramine 10 times as great as those which cause contraction in the normal spleen fail to do so in the denervated spleen and in the spleen of a cat treated with reserpine. Figure 2 illustrates the failure of tyramine to cause contraction in the denervated spleen.

TABLE 4. Threshold amounts causing contraction of the cat spleen

	Normal		Denervated		Reserpine-treated	
	Noradrenaline (μg)	Tyramine (mg)	Noradrenaline (μg)	Tyramine (mg)	Noradrenaline (μg)	Tyramine (mg)
	1·0	—	0·3	> 2·0	0·5	> 4
	2·0	0·3	0·05	4·0	0·1	> 4
	2·0	0·3	0·2		0·05	> 4
	4·0				0·1	
					0·15	
Mean	2·2	0·3	0·18	3·0	0·18	> 4

DISCUSSION

At the beginning of this century both Lewandowsky (1903) and Anderson (1903) investigated the phenomenon called paradoxical pupil dilatation, which had first been described by Budge (1855). This was the dilatation of the pupil after removal of the superior cervical ganglion, which under various conditions was greater than the dilatation of the pupil on the other side where the ganglion was present. Both Lewandowsky and Anderson thought that the greater dilatation on the side without the ganglion was 'due to an increased excitability of the contractile tissues which have been freed from the ganglion', and Lewandowsky expressed this by saying that the ganglion exerted an inhibitory influence on the contractile tissues.

We believe that our observations enable the influence of the ganglion to be described in more detail. So long as the ganglion is present, noradrenaline can be extracted from the iris, and we propose to refer to this as a store. Many assume that the noradrenaline is present in the sympathetic fibres, but there is evidence that the store can be increased by taking up noradrenaline from the blood. The site of peripheral noradrenaline has been discussed by von Euler (1956). When the ganglion is removed the store disappears, and the iris becomes supersensitive to the action of noradrenaline. From these observations it may be concluded that the store is not a totally inactive store, but that it keeps sensitivity low, most probably by continually releasing noradrenaline which then occupies a proportion of the receptors. That there is normally a release of noradrenaline may explain why the store never exceeds a certain amount, and why it diminishes when the nerve degenerates; noradrenaline then continues to be released but the store is not replenished. The failure of tyramine to dilate the denervated pupil is explained by supposing that, normally, tyramine releases noradrenaline from the store, and that tyramine has no action when the store is empty (Burn & Rand, 1958*b*).

We have observed the same changes in the spleen after interruption and degeneration of the post-ganglionic fibres, and have seen that the same events occur in the iris and the spleen after the administration of reserpine. These results leave little doubt that the appearance of supersensitivity after denervation or after administration of reserpine is due to the loss of the store of noradrenaline.

The fall in the amount of noradrenaline which can be extracted from an organ after denervation is evidently a general phenomenon. There is evidence which suggests that a similar fall may occur in isolated organs not previously denervated. Thus Burn & Robinson (1951) found that the perfused rabbit ear was initially less sensitive to noradrenaline than to adrenaline, the former having not more than one-sixth or one-seventh of the constrictor action of the latter. As perfusion continued, the sensitivity to noradrenaline increased from fourfold to tenfold, while the sensitivity to adrenaline increased only slightly.

A similar observation has been made by Walter (1958) in the isolated rabbit iris. He determined the relation between concentration of noradrenaline and dilatation, the iris being suspended in a bath. He found that when he redetermined the concentration–action curve several times the iris became more and more sensitive to noradrenaline, and he suggested that this 'augmentation phenomenon' might be due to the development of 'denervation supersensitivity'. This suggestion was borne out by the observation that if he took irides which had been denervated 2 months previously he failed to observe the augmentation phenomenon. They did not become more sensitive when immersed in the bathing system which he used.

Walter made two other observations which have a parallel in our own work. He saw that denervated irides, when suspended in the bath, not only did not become more sensitive but became less sensitive to noradrenaline, though this diminution in sensitivity did not progress far. Burn & Rand (1958*a*) found that spiral strips of aorta taken from reserpine-treated rabbits were much more sensitive to noradrenaline than strips from normal rabbits. However, the sensitivity of strips from reserpine-treated rabbits was greatest for the first application of noradrenaline, after which the sensitivity declined. We suggest that the decline of sensitivity which Walter observed in his denervated irides and that which we observed in our reserpine-treated aortic strips were due in both cases to the tissue taking up noradrenaline which had been added to the solution in which it was bathed, some receptors being thereby occupied.

The second point was that Walter found that when he denervated the iris on one side, the contralateral iris did not behave like an iris from an unoperated rabbit, but as if it, too, was affected by the denervation. We found that the amount of noradrenaline was less in the normal iris of a cat with one denervated iris than in the normal irides of unoperated cats.

Previous observations by Burn & Robinson (1953) showed that, in the nictitating membrane of the cat, there was a diminution in the amount of amine oxidase after degeneration of the sympathetic fibres. They found a correlation between the fall in amine oxidase and the supersensitivity, the fall being greatest when the supersensitivity was greatest, and suggested that the supersensitivity was due to the fall. However, in twenty out of their twenty-five experiments the amine oxidase in the denervated nictitating membrane was not reduced below 53 % of the amount in the normal membrane, and it is difficult to suppose that such a reduction would be sufficient to change the response of the membrane so greatly as denervation does. We wish therefore to abandon the suggestion of Burn & Robinson, but in doing so must point out that their observations indicate some relation between the store of noradrenaline in the wall and the amount of amine oxidase which is present. That denervation causes changes in amine oxidase has been shown by others, notably by Strömblad (1956*a*), who has made careful studies of the effect of denervation on the enzyme content of the salivary glands. He demonstrated the presence of amine oxidase in both the submaxillary and parotid glands of the cat, and found that section of the chorda tympani caused a fall in the amine oxidase of the submaxillary gland. Degeneration of the post-ganglionic parasympathetic fibres caused a fall in the amine oxidase of the parotid gland. Excision of the superior cervical ganglion, however, failed to diminish the amine-oxidase activity in the salivary glands, and in the parotid gland it even caused a rise. These observations alone show that the relation of denervation to amine oxidase is obscure.

In view of the evidence that tyramine acts by releasing noradrenaline from a store in organs with sympathetic innervation (Burn & Rand, 1958*b*) it is interesting to note that Strömblad (1956*b*) found that tyramine became inactive in the submaxillary gland after excision of the superior cervical ganglion. Von Euler & Purkhold (1951) showed that the store of noradrenaline in the submaxillary glands of the sheep became very small after extirpation of the superior cervical ganglion.

SUMMARY

1. The supersensitivity to noradrenaline and adrenaline which develops in organs innervated by sympathetic nerves after degeneration of these nerves is attributed to the disappearance of a store of noradrenaline in those organs.

2. The iris of the cat's eye, and the spleen of the cat both contain noradrenaline. After degeneration of the sympathetic nerves this store of noradrenaline in both these organs declines, and at the same time sensitivity to noradrenaline greatly increases.

3. The failure of tyramine to act on the denervated iris and spleen is explained by the fact that tyramine acts in the normally innervated organ by releasing noradrenaline from the store.

4. The changes produced in the iris and spleen of the cat by degeneration of sympathetic fibres are also produced by treatment of the animal with reserpine.

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HISTAMINE, 5-HYDROXYTRYPTAMINE AND SERUM GLOBULINS IN THE FOETAL AND NEO-NATAL RAT

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Acute inflammation following experimental injury in rat foetuses up to 18½ days old differs strikingly from that in the adult (Dixon, unpublished). The blood vessels appear inert and no inflammatory oedema develops.

In the adult rat, histamine, 5-hydroxytryptamine (5-HT) and certain serum globulins are known to modify capillary permeability (Parratt & West, 1957*b*, 1958; Spector, 1956, 1957; Spector & Willoughby, 1957*a*, *b*, 1958). The relative importance of these three mediators may vary from one tissue to another. However, it appears that in turpentine-induced pleurisy at least, histamine, possibly in conjunction with 5-HT, is responsible for the increase in capillary permeability which develops during the first hour, after which the globulin system takes over (Spector & Willoughby, personal communication).

The anomalous behaviour of foetal capillaries could be explained by a deficiency of one or more of these mediators in the tissues. Their levels were therefore investigated in foetuses of various ages and an attempt was made to correlate the histamine with the development of the mast cells, which are a rich source of histamine in the adult (Riley, 1953; Riley & West, 1953, 1955; Parratt & West, 1957*a*, *d*; Mota, Beraldo, Ferri & Junqueira, 1954).

METHODS

Rats of the Wistar strain weighing 150–250 g before pregnancy were used. Unstained vaginal smears were examined daily and each female in oestrus was placed during the night in a separate cage with a male. Pregnancy was considered to begin in the middle of the night in which mating took place; it lasted 21½–22½ days.

Young rats which were used for preparing extracts were killed with ether and bled by decapitation. In order to remove the foetuses for extraction from a pregnant rat, it was killed by a blow on the head under light ether anaesthesia, the abdominal wall and uterus were rapidly opened, the foetuses were freed from their membranes and allowed to bleed from the cords. In some experiments in which 20½-day-old foetuses were removed they were allowed to survive for 4–5 hr before they were used for extraction. In these experiments each umbilical cord was tied tightly with 'Mersilk' as closely as possible to the abdominal wall and then severed distal to the ligature. The foetuses were laid on cotton-wool moistened with normal saline in an incubator at 35° C, and they were stroked frequently with a damp cotton-wool swab to stimulate respiration. This became spontaneous and regular after 30–40 min and they could then safely be left alone.

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The extraction and assay of histamine. Histamine was extracted by the method of Feldberg & Talesnik (1953). The whole animals or various tissues were lightly blotted, weighed, ground with N-HCl 2 ml./g and sand in a mortar, and diluted with distilled water 10 ml./g. After further grinding the pulp was washed with 5–10 ml. distilled water into a conical flask, brought to the boil and filtered through cotton-wool. Extracts were stored up to 2 weeks before assay. Several extracts were tested at intervals for histamine loss, but none occurred.

The extracts were neutralized with N-NaOH to approximately pH 7 immediately before assay, which was performed on an atropinized guinea-pig ileum suspended in an 18 ml. bath of oxygenated Tyrode solution at 37° C. All histamine values refer to the base. The active principle was shown to be histamine, using mepyramine (0·05 μg/ml.). The extracts of liver and sometimes of intestine and lung contained another smooth-muscle stimulant which was not antagonized by brom L.S.D. (BOL 148) (0·005 ml./ml.). If this happened, contractions equivalent to 0·02 μg or more histamine base were subtracted from the values obtained during the assay.

The extraction and assay of 5-HT. 5-HT was extracted by the method of Amin, Crawford & Gaddum (1954). Whole foetuses and young rats were weighed, chopped finely with pure acetone, 20 ml./g, and allowed to stand with occasional stirring at room temperature for 1 hr. The acetone was filtered off and replaced by 20 ml./g 95% acetone. After 30 min the acetone was decanted and the combined filtrates evaporated under reduced pressure at 35° C until completely dry. Extracts were stored at −10° C during the night before assay. The extracts were taken up in 1–5 ml. of a salt solution containing (g/l.): NaCl 9, KCl 0·4, $CaCl_2$ 0·03, $NaHCO_3$ 0·15, glucose 1·0. Estimations were carried out on an atropinized rat colon suspended in an oxygenated 18 ml. bath of the same solution at 20° C. All 5-HT values refer to the base. The active principle was shown to be 5-HT with BOL 148 (a 5-HT antagonist) and by tachyphylaxis using large doses of 5-HT.

The mast cell content of skin. At every stage mast cells were counted in a minimum of 16 random fields under a 1/6 in. (4·2 mm) objective. They were taken from two or more of the following skin areas—neck, back, rump and abdomen, in at least two animals. The pieces of skin were fixed in a mixture of 10 ml. formalin, 90 ml. 95% alcohol and 1 g Ca acetate (Smith & Atkinson, 1956). They were washed in two changes 95% alcohol (1 hr each) and processed in the normal way. Paraffin sections were cut at 5 mμ, taken to 95% alcohol and left for 1 hr in the following solution at room temperature: 100 ml. 70% alcohol, 0·5 ml. concentrated HCl, and 0·25 g toluidine blue. Excess dye was removed by rinsing in 95% alcohol containing 0·5% HCl. Sections were counterstained with 0·01% eosin in 95% alcohol, cleared and mounted.

Assessment of the 'permeability factor' content of sera. Blood was collected from the posterior venae cavae of adult rats under ether anaesthesia and from the necks of decapitated foetuses and young rats. Undiluted sera and 1:10 dilutions which had stood at room temperature for 1 hr were injected intradermally into the shaved abdominal skin of adult rats which had received trypan blue intravenously immediately before. The intensity of blueing was assessed subjectively after 20 min.

In some foetuses and young rats histaminase was also estimated. The method used was the volumetric one of Kapeller-Adler (1951)

RESULTS

Histamine levels

The results obtained on whole foetuses and young rats are shown in Fig. 1. Two or more animals per litter were extracted at each stage, and the points represent the mean values from at least two litters.

Histamine is first detected in foetuses on the 11th day of gestation. It increases tenfold during the next 48 hr and then remains fairly constant in relation to body weight from the 13th to the 20th days. Before parturition a second rise begins and histamine accumulates rapidly after birth, reaching a maximum between the 6th and 8th hours. Some of this histamine is immediately lost, for the peak is followed by a sharp drop. At 24 hr the content equals that shortly before birth, but by the second day it increases again and this build-up continues until the 3rd to the 5th day. From then until the 7th day, when estimations were discontinued, the level remains roughly constant.

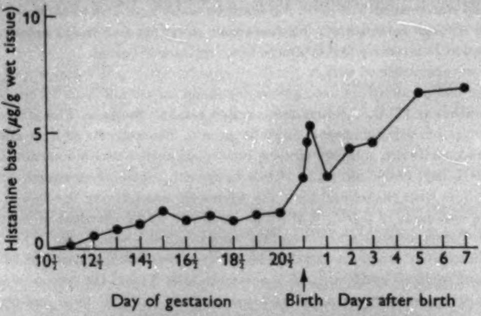

Fig. 1. Histamine content of whole foetuses and young rats.

Table 1. Histamine content of five tissues from foetuses

Day of pregnancy	Histamine (μg/g wet tissue)				
	Skin	Lung	Liver	Intestine	Muscle
18½	0·9	0·9	6·6	1·6	1·2
19½	2·0	0·7	5·8	1·3	1·5
20½	4·0	0·5	2·0	0·8	1·3
21½	5·5	1·2	6·5	1·3	1·8

Table 2. Histamine content of five tissues from young rats

Hours after birth	Histamine (μg/g wet tissue)				
	Skin	Lung	Liver	Intestine	Muscle
3	8·9	6·3	7·2	8·0	5·1
6	9·1	4·5	5·2	3·2	2·9
24	18·8	2·3	1·7	3·2	3·9
48	14·4	0·4	0·3	0·8	3·1
72	15·7	0·6	0·4	0·7	2·7
120	22·2	0·5	0·3	0·6	3·5
168	17·3	0·7	0·2	0·6	2·7

Histamine was assayed in five tissues between the 18th day of gestation and 7 days after birth. Two litters were studied at every stage, and samples were collected from a minimum of three animals in each. The means of the results appear in Tables 1 and 2.

The histamine contents of lung and intestine follow the same pattern as in the whole foetus. They are low and roughly constant during pregnancy. Immediately after birth they climb rapidly and reach a maximum about the third hour post-partum. During the following 24–48 hr there is a steady decline to low levels which are maintained to the end of the first week. The behaviour of muscle is similar to that of lung and intestine but the post-partum rise is smaller and the histamine content decreases slowly and erratically during the first 7 days after birth.

TABLE 3. Histamine content of foetuses 4–5 hr after removal from the uterus on the 20th day of gestation

Hours after delivery	Histamine (μg/g wet tissue)
4	9·3; 12·8; 13·4
5	7·3; 11·4

TABLE 4. Histamine content of five tissues from foetuses 4–5 hr after removal from the uterus on the 20th day of gestation

Hours after delivery	Histamine (μg/g wet tissue)				
	Skin	Lung	Liver	Intestine	Muscle
4	8·5	7·7	3·4	4·3	3·8
5	4·5	4·0	4·1	2·5	2·4

The antepartum levels of liver vary, but the content at birth is high. This is maintained for the first few hours post-partum and then falls precipitously to a low level which does not alter after the second day.

The skin differs strikingly from the other tissues. Histamine increases steadily during the 3 days before delivery, and then soars in the next 24 hr to over three times the birth value. It fluctuates thereafter around this high level.

In order to find out whether separation from the placental circulation coupled with exposure to the atmosphere and the breathing of air could accelerate the rise in histamine which normally begins on the 20th day, foetuses were removed at this time and allowed to survive for 4–5 hr. They yielded at least five times the average histamine value of 20½-day-old foetuses and exceeded the values obtained for baby rats 4–6 hr after normal birth. The histamine values obtained in these foetuses are given in Table 3.

In these foetuses which had survived for 4–5 hr the histamine was also estimated separately in lung, intestine, muscle and skin. The mean values are shown in Table 4. All values are higher than the corresponding values for normal 20½-day-old foetuses which are given in Table 1.

The mast-cell content of skin

The dermal mast cells at 17½ days gestation are relatively scanty, small and poor in granules; they become steadily more numerous and a little larger during the next 2½ days, but between the 20th and 21st days the number of cells is trebled and the average size and depth of staining increase strikingly.

During the first few hours after birth approximately 70% of the cells degranulate. Since the majority of these stain feebly and are barely recognizable as mast cells, the total count is reduced, but by 24 hr post-partum both the numbers of granules and of cells are restored. Throughout the following 6 days the cell count fluctuates a little although no significant change occurs in the staining. There is a striking correlation between mast cell count and histamine content of skin (Fig. 2); the main increases and decreases in the cell count are followed 24 hr later by similar changes in histamine content.

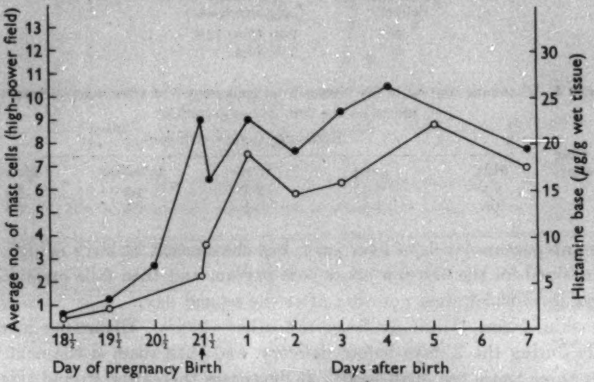

Fig. 2. Histamine (○—○) and mast cell (●—●) contents of skin of foetal and young rats.

5-*HT levels*

By the methods of assay employed 5-HT was first detectable in foetuses at 15½ days gestation. The content then increased steadily in proportion to body weight up to birth and tended to reach an equilibrium after several fluctuations at 4 days post-partum. These variations might have been reduced if a larger series of animals had been used. The results are illustrated in Fig. 3, each point of which represents the mean value for at least four animals from two different litters.

No *histaminase* was detected in foetuses aged 20½ and 21½ days and in rats between birth and one week old.

The '*permeability factor*' content of sera was approximately the same in the sera of 20½-day-old foetuses and of 7-day-old and adult rats. This is shown in Table 5.

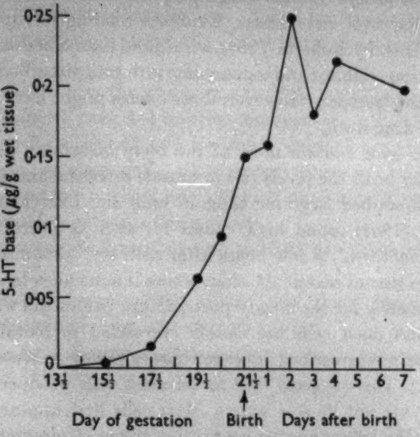

Fig. 3. The 5-HT content of whole foetuses and young rats.

Table 5. The effect of sera from rats of different ages on capillary permeability

Age	Intensity of 'blueing' of injection sites: Undiluted serum	Serum diluted 1:10 with 0·9% saline
20½-day-old foetuses	+ + + +	+ +
7 days post-partum	+ + + +	+ + +
Adult	+ + + +	+ + +

+ +, + + +, + + + + = degree of increased capillary permeability.

DISCUSSION

The total body histamine of the foetus is more than doubled during the last 24 hr *in utero*, but its origin is obscure. Diffusion across the placenta from the maternal blood is unlikely in view of the extremely high histaminase content of the rat placenta (Ahlmark, 1944; Swanberg, 1950; Roberts & Robson, 1953). A more probable explanation is decarboxylation of L-histidine in the foetus. Schayer (1956*a*, *b*) believes that all histamine bound in mammalian tissues is produced *in situ* by this method.

Histamine synthesis by foetuses is accelerated if they are taken prematurely from the uterus. Foetuses removed on the 20th day of gestation synthesize approximately 36 µg histamine during the first 3–6 hr after removal compared with 11 µg which they would produce in the last 24 hr if left *in utero*. This difference may result from the fact that exposure to external conditions accelerates synthesis; or alternatively, the normal synthesis is greater than appears from the assays because a high proportion of the histamine is rapidly destroyed by placental histaminase. Evidence tending to support this hypothesis was obtained by Roberts (1954), who found that subcutaneous injections of the histaminase inhibitor aminoguanidine into rats disturbed pregnancy and killed some of the foetuses. However, these effects might have been due to the toxicity of the drug itself.

The skin and liver contain most of the body histamine during gestation, but shortly after birth the levels rise in muscle intestine and lung. Parratt & West (1957*c*) described large increases in lung and liver histamine at birth and Hardwick (1954) found high values for skin at parturition compared with those for foetuses. A few hours after delivery, histamine begins to decrease in all the tissues except the skin, where it continues to accumulate and is largely responsible for the steady post-partum rise in the whole animal.

Histamine and mast cells are closely correlated in foetal and neo-natal rat skin, as they are in various adult rat tissues (Riley & West, 1953; Parratt & West, 1957*c*). This suggests that foetal mast cells produce or store histamine, but they are not its sole source. Mast cells first appear in rat foetuses 1·4 cm long in the subcutaneous connective tissue (Holmgren, 1946), i.e. the 16th day of gestation (Wintrobe & Schumacker, 1936), but histamine is detectable on the 11th day.

It is not clear how histamine is lost after the peak at 3–6 hr after birth. It cannot be due to histaminase, which Schayer (1956*a*) claims is the only major histamine-destroying enzyme in adult rats, since foetuses do not contain it (Roberts & Robson, 1953) and it does not develop during the first week post-partum. Histamine may be excreted in the urine either free or after acetylation.

5-HT rises steadily and rapidly in proportion to the body weight from the 15th day of gestation to the 4th day post-partum without a sudden increase at birth. This finding substantiates that of Parratt & West (1957*d*). It would be interesting to know whether the skin 5-HT is correlated with its mast-cell population at different stages of pregnancy, in view of the recent evidence that the mast cells of adult rats contain 5-HT (Benditt, Wong, Arase & Roeper, 1955; Bhattacharya & Lewis, 1956*a*, *b*; Rowley & Benditt, 1956; Parratt & West, 1957*c*).

SUMMARY

1. The histamine contents of whole rats were determined between 10 days gestation and 7 days after birth, and the contents of lung, intestine, muscle, liver and skin between 18 days gestation and 7 days post-partum.

2. Low histamine contents were found in whole foetuses from the 11th to the 20th days but the content increased from the 21st day to the 4th day post-partum. The rise was steady, apart from a peak 6–8 hr after birth followed by a sharp fall lasting 16–18 hr.

3. Lung, intestine and muscle showed similar post-partum peaks in histamine content which was otherwise low. Skin histamine rose steadily from 18½ days gestation to 5 days after birth. Liver histamine was fairly high before birth but low after.

4. Foetuses taken from the uterus on the 20th day of gestation showed an exaggerated histamine peak 4–5 hr after removal.

5. In skin, mast-cell count and histamine content are closely correlated between 18½ days gestation and 7 days post-partum.

6. The 5-HT content of whole rats increases steadily until the 4th day post-partum and thereafter remains fairly steady.

7. No histaminase was found between the 20th day of gestation and 7 days after birth.

8. The content of 'permeability factor' was about the same in sera of 20½-day-old foetuses and 7-day-old and adult rats.

It is a great pleasure to thank Sir Roy Cameron, F.R.S., for his advice and encouragement. This work was aided by a grant from the Agricultural Research Council.

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THE UPTAKE OF 5-HYDROXYTRYPTAMINE BY BLOOD PLATELETS IN THE COLD

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At 37° C human blood platelets are able to take up 5-hydroxytryptamine (5-HT) against concentration gradients of up to 1000:1, and it has recently been shown (Born & Gillson, 1959) that this uptake has some of the characteristics of active transport which have been discussed by Rosenberg (1954). This paper describes experiments which show that platelets take up 5-HT at 0–2° C, although much less than at 37° C. The results indicate that the uptake in the cold is due to the diffusion of 5-HT into the platelets, and that some 5-HT is adsorbed in or on the platelets. Part of this work has been communicated to the Physiological Society (Born & Bricknell, 1959).

For these experiments 5-HT was added to plasma rich in platelets which was centrifuged after incubation. The platelets formed a pellet in the bottom of the centrifuge tube, from which as much plasma as possible was removed. A little plasma, however, inevitably remained in the platelet pellet. Since this plasma contained 5-HT its volume had to be measured before the 5-HT content of the platelets in the pellet could be obtained. This was done by adding either inulin or labelled serum albumin to the plasma before centrifugation. These substances are not taken up by platelets; therefore the amounts found in the platelet pellet provided a measure of the volume of plasma in the pellet.

METHODS

The methods described by Born & Gillson (1959) were used to obtain from healthy persons citrated plasma rich in platelets and for counting the platelets. Samples of the plasma were pipetted into centrifuge tubes made of cellulose acetate and with a capacity of 3 ml. The tubes stood in a mixture of ice and water at 0–2° C.

Platelets were isolated from the plasma by centrifuging the tubes at 15,000 *g* for 5 min at 0–1° C; this was done in the swing-out head used with the high-speed attachment of an MSE refrigerator centrifuge. The supernatant plasma was free from platelets. It was decanted and retained; the platelets remained as a pellet in the bottom of the tube. The tubes were inverted and remained standing upside down on filter paper for about 5 min in order to drain as much plasma as possible away from the platelet pellet. Then the inside wall of the tube, down to just above the pellet, was wiped dry with filter paper.

Uptake of 5-HT. In order to determine the uptake of 5-HT by platelets, known amounts of

5-HT labelled with ^{14}C were added to the plasma before centrifugation and the platelet pellet was dispersed in 0·4 ml. water. The suspension was washed into a glass-stoppered test-tube with 0·8 ml. water and acetone was added to make the volume 10 ml. After shaking the tube was left at −16° C during the night. The tube was then centrifuged to precipitate the proteins and samples of the clear supernatant solution were dried on planchettes in duplicate. The radioactivity of the dried material was determined at infinite thinness in a helium gas-flow counter which had an apparent counting efficiency of about 50%. In order to determine the plasma concentration of 5-HT 1 ml. of the same platelet-free plasma was mixed with acetone to make a volume of 10 ml. The preparation of the acetone extract and the subsequent measurement of its radioactivity were the same as with the platelets. Control experiments showed that under the conditions used 5-HT was not broken down, and that the procedures described extracted all the 5-HT present in platelets and in plasma.

Inulin space. In order to determine the volume of plasma in the platelet pellet its inulin or albumin space was determined as follows.

Platelet-rich plasma was thoroughly mixed with inulin. The platelet pellet obtained after centrifugation was dispersed in 0·8 ml. distilled water with a glass homogenizer (Potter & Elvehjem, 1936). 0·5 N-NaOH 0·5 ml. and $ZnSO_4$ solution (10% w/v) 0·5 ml. were added to precipitate the proteins, and after mixing the tube was centrifuged. The clear supernatant was transferred into a graduated test-tube. The precipitate was stirred with water, centrifuged, and the clear supernatant was added to the first supernatant. The volume was made up to 2·0 ml. with water, and the concentration of inulin in this solution was determined by the method of Bacon & Bell (1948). In order to determine the plasma concentration of inulin the same platelet-free plasma was diluted to 100 ml. with water and 2·0 ml. was mixed with 0·5 ml. NaOH and 0·5 ml. $ZnSO_4$ solutions, as described above. After centrifugation, 1·0 ml. of the clear supernatant was added to 1·0 ml. water and the inulin concentration of this mixture was determined.

In each experiment a sample of platelet-rich plasma containing no inulin was put through the same procedure to obtain blank values. The colorimetric readings obtained in this way with platelets and with plasma were subtracted from the corresponding readings found in the presence of inulin. These blank values were very low.

Albumin space. The procedure was essentially the same as that used for determining the inulin space. The platelet pellet obtained after centrifuging the plasma was, however, dispersed in 0·4 ml. water and the volume of the suspension was made up to 10 ml. with saline. This suspension was poured into a liquid counter, type M6, with a capacity of 10 ml. and the radioactivity of the ^{131}I present was determined. One millilitre of the corresponding platelet-free plasma was diluted to 100 ml. with saline and the radioactivity in 10 ml. of this dilution was measured.

The substances used were dissolved in physiological saline.

5-*Hydroxytryptamine* labelled with ^{14}C (^{14}C-5-HT) was supplied as the creatinine sulphate by the Radiochemical Centre at Amersham; it had a specific activity of 6·87 mc/mM.

Human serum albumin labelled with ^{131}I was also supplied by the Radiochemical Centre; it was in a solution containing 20 mg albumin/ml. saline.

Inulin was kindly given by Dr R. B. Fisher; it was purified by two precipitations in 80% (v/v) ethanol, followed by washing with distilled water.

RESULTS

Comparison of uptake of ^{14}C-5-HT at 0–2° C and at 37° C

At 37° C platelets take up 5-HT from plasma until its concentration in the platelets is up to one thousand times greater than it is in the plasma (Born & Gillson, 1959). The amount of 5-HT present in plasma in the pellet is so small compared with the amount in the platelets that it can be neglected when the concentration of 5-HT in the platelets is calculated.

At 0–2° C platelets also take up 5-HT. However, as is shown in Table 1, at 37° C the platelets took up 27–42 times more ^{14}C-5-HT than at 0–2° C. Since the volume of 10^8 human platelets is approximately 1 μl. (Tocantins, 1938; Hardisty & Stacey, 1955; Born & Gillson, 1959), these results show that even at the lower temperature the concentration of ^{14}C-5-HT exceeded that in the plasma. This could be either because the ^{14}C-5-HT was taken up or because it exchanged with non-radioactive 5-HT present in the platelets.

TABLE 1. Uptake of ^{14}C-5-HT by human platelets from plasma at 0–2° C and at 37° C

			Concentration of ^{14}C-5-HT in platelet pellet after 60 min (ng/10^8 platelets)		
Experiment	Concentration of ^{14}C-5-HT in plasma (ng/μl.)	Temperature (° C)	Uncorrected for plasma in pellets	Corrected for plasma in pellets	Difference (%)
1	0·4	0–2	3·6	3·5	3
	0·4	37	—	96	—
	4·0	0–2	10·9	9·9	10
	4·0	37	—	316	—
2	0·4	0–2	2·1	2·0	5
	0·4	37	—	84	—
	4·0	0–2	12·8	11·7	10
	4·0	37	—	341	—

When the uptake of ^{14}C-5-HT in platelets maintained at the lower temperature was calculated, it was necessary to correct for the amount of ^{14}C-5-HT present in plasma in the pellet. To make this correction the volume of plasma which remained in the platelet pellet after centrifugation was determined by estimating the inulin and serum albumin spaces in the pellet.

Inulin and albumin spaces in the platelet pellets

When inulin and labelled albumin were added to platelet-rich plasma before centrifugation they were found in the pellet of platelets. From the concentrations of inulin and labelled albumin in the plasma and the amounts found in the pellet, the volume of plasma in the pellet was calculated. This volume was called the inulin space and the albumin space and was expressed as μl. plasma/10^8 platelets in the pellet.

The results of twelve determinations of the inulin space were remarkably constant; the mean was 0·352 μl. plasma/10^8 platelets with a standard error of $\pm 0{\cdot}005$. The magnitude of the inulin space was not significantly affected by the time that passed between mixing inulin with plasma and centrifuging, nor by the concentration of added inulin. The 'albumin space' in the platelet pellets was found to be of the same order. The mean value of 6 determinations was $0{\cdot}402 \pm 0{\cdot}030$ μl. plasma/10^8 platelets. This value was not significantly affected by the concentration of added albumin, which varied over a tenfold range in different experiments. The results obtained with inulin and albumin support the assumption that the inulin and labelled albumin found in platelet pellets was present in plasma and not in platelets. It is therefore concluded that the inulin and albumin space represents the volume of plasma in the platelet pellet. Table 1 shows that, with a 5-HT concentration of 4 ng/μl. plasma, about 10% of the ^{14}C-5-HT found in platelet pellets was in the plasma and not in the platelets.

'5-HT space' in platelet pellets

When experiments similar to those with inulin and albumin were made with 5-HT the results were different. First, the amount of ^{14}C-5-HT found in platelet pellets increased as the time between adding the amine and centrifuging the plasma increased, for about 60 min; this is shown in Fig. 1.

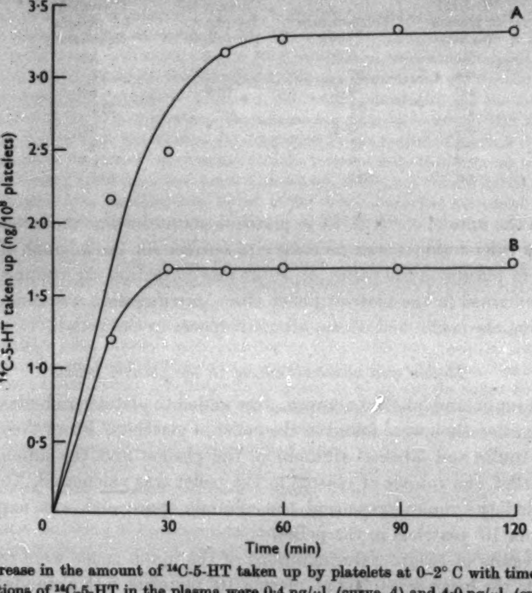

Fig. 1. Increase in the amount of ^{14}C-5-HT taken up by platelets at 0–2° C with time. The concentrations of ^{14}C-5-HT in the plasma were 0·4 ng/μl. (curve *A*) and 4·0 ng/μl. (curve *B*).

Secondly, the apparent '5-HT space', expressed in the same way as the inulin or albumin space, depended on the concentration of added 5-HT in the plasma. To make sure that the amount of ^{14}C-5-HT recoverable from platelet pellets was not limited by time, the ^{14}C-5-HT was mixed with platelet-rich plasma in tubes which were kept at 0–2° C for 60 min and shaken every few minutes before being centrifuged. Figure 2 shows that the '5-HT space' decreased with increasing concentrations of 5-HT in the plasma. However, when the concentration exceeded about 2 ng/μl. the 'space' reached a constant minimum value of 1·0–1·4 μl./10^8 platelets.

When the amount of ^{14}C-5-HT in the plasma of the platelet pellet, calculated on the basis of the inulin space, was subtracted from the total amount of ^{14}C-5-HT in the pellet, the result showed again that the uptake of 5-HT by the platelets depended on the concentration of 5-HT in the plasma. In Fig. 3 the concentration of ^{14}C-5-HT in the platelets, corrected for the ^{14}C-5-HT present in the plasma of the pellet, has been plotted against the concentration of ^{14}C-5-HT in the plasma. The figure shows that, as the concentration in the plasma increased, the amount of 5-HT associated with the platelets increased as well. With concentrations of less than 2 ng/μl. the increase of ^{14}C-5-HT in the platelets was greater than that in the plasma; with higher concentrations the increase was directly proportional. From the experiments shown in Fig. 3

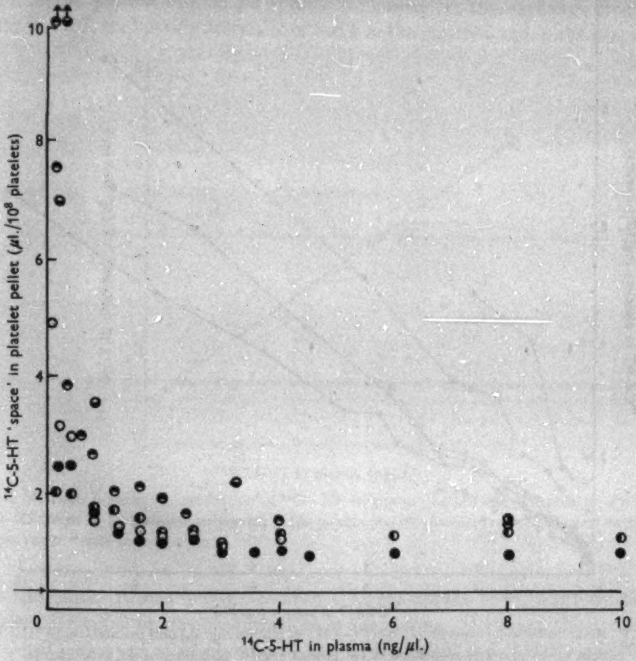

Fig. 2. Relation between concentration of ^{14}C-5-HT in the plasma (ng/μl.) and the size of the '5-HT space' in the platelet pellet: results of six experiments. For definition of '5-HT space' see text. The line indicated by the arrow shows the inulin space.

the average amount of ^{14}C-5-HT taken up by platelets at 0–2° C was calculated for different concentrations of ^{14}C-5-HT in plasma. In Fig. 4 these mean values are plotted against the plasma concentrations. Using these points a regression line was calculated which had the equation, $y = 0{\cdot}72x + 0{\cdot}93$.

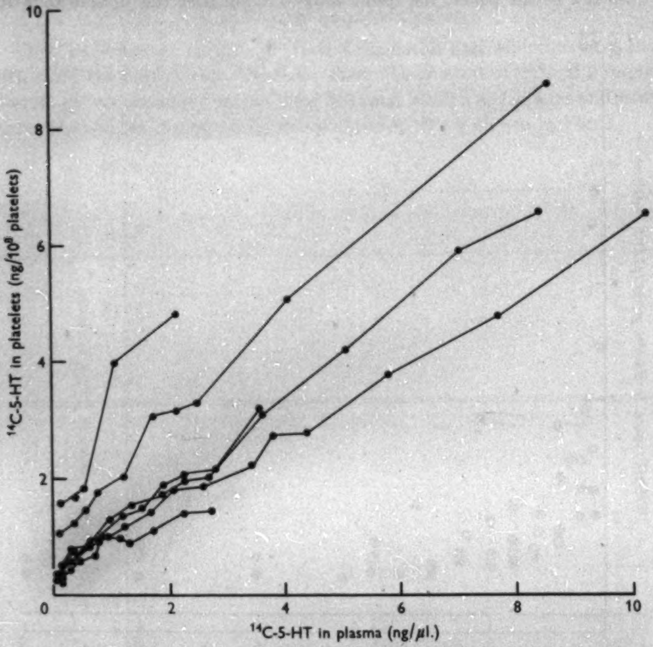

Fig. 3. Relation between concentration of ^{14}C-5-HT in plasma (ng/μl.) and the amount of ^{14}C-5-HT taken up by platelets suspended in the plasma (ng/10^8 platelets) in six experiments.

DISCUSSION

The experiments have shown that human platelets take up small amounts of 5-HT in the cold; the amounts taken up were 1–10 ng/10^8 platelets. Platelets contain about 50 ng 5-HT/10^8 platelets (Born & Gillson, 1959). Although it is possible to determine as little as 1 ng 5-HT by bioassay (Vane, 1957) the smallness of the increases in the 5-HT content of the platelets would tend to make the determinations inaccurate. Only by using radioactive 5-HT was it possible to make the observations recorded in this paper.

The use of radioactive 5-HT raised the problem, however, whether the uptake of ^{14}C-5-HT might not represent an exchange of radioactive for non-radioactive 5-HT in the platelets, rather than an increase in their content of 5-HT. Although the possibility that such an exchange occurs has not been excluded, it seems inadequate to explain the observed uptake of ^{14}C-5-HT, for the following reason. The exchange of radioactive for non-radioactive molecules of 5-HT would be expected to depend not on the concentration of 5-HT in the plasma but on its specific radioactivity; the exchange should continue until the specific activities of 5-HT in the platelets and in the plasma were the same. It was found, however, that the uptake of ^{14}C-5-HT by the platelets depended on its concentrations in the plasma. Moreover, the uptake of ^{14}C-5-HT stopped after about 1 hr and it was calculated that the specific activity of 5-HT in the platelets remained well below that in the plasma. These considerations suggest that the uptake of labelled 5-HT represents a true increase in the amount of 5-HT in the platelets.

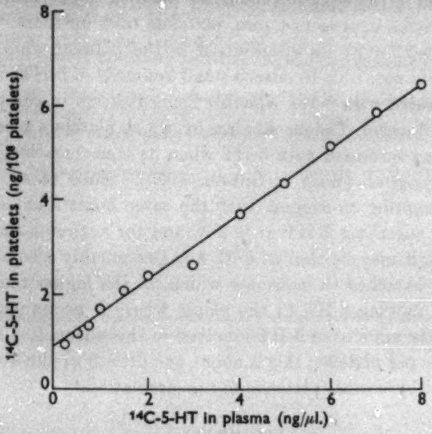

Fig. 4. Relation between concentration of ^{14}C-5-HT in plasma (ng/μl.) and the amount of ^{14}C-5-HT taken up by platelets suspended in the plasma (ng/10^8 platelets): average values for the six experiments shown in Fig. 3.

The finding that the amount of 5-HT taken up at 37° C is much greater than at 0–2° C favours the suggestion that at 37° C 5-HT is actively transported into platelets (Born & Gillson, 1959). The mechanism by which small amounts of radioactive 5-HT become associated with the platelets at 0–2° C is presumably different.

The relation between the concentration of 5-HT in the plasma (x) and the average amount taken up by the platelets (y) is given by the equation, $y = 0{\cdot}72x + 0{\cdot}93$, in which x represents ng ^{14}C-5-HT/μl. plasma and y

ng ^{14}C-5-HT/10^8 platelets. Since the volume of 10^8 platelets is approximately 1 μl., y also represents ng/μl. platelet volume. In view of this it is possible to suggest a physiological meaning for the parameters of the equation. About 75% of the constituents of the platelets is water; the coefficient of 0·72 suggests, therefore, that the ^{14}C-5-HT distributed itself in the platelet water. If this is so, it follows that 5-HT is able to diffuse into platelets, but the diffusion is slow; at 0–2° C it continued for about an hour.

The term 0·93 in the equation indicates that, as the concentration of ^{14}C-5-HT in the plasma approached zero, platelets take up more ^{14}C-5-HT than can be accounted for by its distribution in the platelet water. This shows that the platelets were able to adsorb small amounts of 5-HT. The adsorbing sites were saturated with 5-HT when its concentration in the plasma was in the region of 1·0 μg/ml. The mechanism by which platelets accumulate 5-HT at 37° C becomes saturated with 5-HT when its concentration in the plasma exceeds 0·5–1·0 μg/ml. (Born & Gillson, 1959). Since these values are so similar it is tempting to suggest that the same constituent of platelets is responsible for adsorbing 5-HT at 0–2° C and for accumulating it at 37° C. In other words, it may be that at 0° C, and presumably also at 37° C, 5-HT becomes firmly attached to molecules which, at the higher temperature, are responsible for moving 5-HT to the places where it accumulates. One can calculate that the amount of 5-HT involved in this adsorption is of the order of 10^4 molecules per platelet; this is about one-fiftieth of the average amount of 5-HT carried by normal platelets in the circulation.

SUMMARY

1. Experiments were made to determine whether human platelets take up 5-hydroxytryptamine when they are suspended in citrated plasma at 0–2° C.

2. Platelets took up 5-hydroxytryptamine labelled with ^{14}C (^{14}C-5-HT) but the uptake at 0–2° C was 27–42 times less than at 37° C; the amount taken up at 0–2° C increased with time for about 60 min.

3. The amount taken up at 0–2° C increased with the concentration of ^{14}C-5-HT in the plasma. With concentrations of less than 2 ng/μl. plasma, platelets appeared to adsorb ^{14}C-5-HT. With higher concentrations the amount taken up increased in proportion to the plasma concentration. The average amount (y) taken up in six experiments depended on the concentration (x) of ^{14}C-5-HT in the plasma as follows: $y = 0{\cdot}72x + 0{\cdot}93$, where y is expressed in ng ^{14}C-5-HT/10^8 platelets, and x in ng ^{14}C-5-HT/μl. plasma.

4. Since 10^8 platelets have a volume of about 1 μl., and since about 75% of the platelets is water, the coefficient of 0·72 suggests that ^{14}C-5-HT was able to diffuse into platelets and to distribute itself in the platelet water.

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MUSCLE FATIGUE AND RECOVERY FROM FATIGUE INDUCED BY SUSTAINED CONTRACTIONS

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Previously reported experiments (Clarke, Hellon & Lind, 1958) have disclosed that the durations of submaximal sustained contractions are profoundly influenced by the temperature of the contracting muscles. Contractions are held longest when the muscle temperature, measured half way between the skin and the centre of the arm, was about 27° C (i.e. after 30 min immersion in water at 18° C), above and below which temperature the durations of contractions fall. In those experiments, the forearm was immersed in water throughout the experiments and the interval between the five successive, sustained contractions remained constant at 20 min. In all experiments where the muscle temperature was 27° C or above, the durations of the successive contractions fell from the initial value to a lower, steady level by the fourth and fifth contractions. Clearly, even though 20 min were allowed between contractions, this period was insufficient to allow full recovery of the function of the muscles.

It is well known (e.g. Vernon, 1929) that changes in the interval between successive contractions or successive bouts of rhythmic contractions vary the tension that can be exerted and the duration of such contractions. Nothing, however, is known about the effect that variations of the muscle temperature have on these changes. One of the aims of the present experiments was to determine whether the influence of muscle temperature, in reducing the duration of sustained contractions previously demonstrated at muscle temperatures of 27° C and above (Clarke *et al.* 1958), is maintained when the interval between contractions is varied. This experimental intention, where the intervals between contractions were varied between 3 and 40 min when the forearm was immersed in water at two temperatures, seemed additionally to offer the opportunity of studying the inter-relationship of these variables on the process of recovery. Some of these experiments have already been briefly reported (Lind & Samueloff, 1957).

METHODS

The apparatus comprised a simple hand-grip, strain-gauge dynamometer, previously described (Clarke *et al.* 1958), mounted on a rigid pillar which ran on castors to prevent the effective use of muscles other than the flexors of the forearm in making the contractions. The upper arm was held at an angle of approximately 90° to the forearm in all contractions.

Four healthy young men acted as subjects; they were fully trained in the use of the apparatus and accustomed to holding sustained submaximal contractions to the agreed end-point, which was the failure to maintain the required tension. Some discomfort was experienced towards the end of such contractions but experiments repeated in the same conditions yielded results with a coefficient of variation of 3·64%; this was considered to be satisfactory evidence that the subjects maintained the contractions to the agreed end-point, and were not influenced by accompanying ischaemic discomforts, which varied in intensity from experiment to experiment. No subject performed more than one experiment in any 24-hr period.

At the start of each experiment two maximal contractions were made, and subsequent sustained contractions were held at 1/3 of the average of these contractions. After making the maximal contractions the forearm was immersed in a water-bath at 18 or 34° C, maintained to $\pm 0{\cdot}5^\circ$ C, for the remainder of the experiment. Thirty minutes after immersion, the first of five successive sustained contractions was made. The remaining four contractions followed, with an interval from the end of one contraction to the beginning of the next of 40, 20, 7 or 3 min; this interval remained constant in any one experiment.

Since the required submaximal tension can no longer be held at the end of a sustained contraction, at this time the ability of the muscle to exert maximum tension has decreased to this value. To estimate the rate of recovery of the ability to exert maximum tension, another series of experiments was carried out, where two brief maximum efforts (each < 3 sec, with a 15 sec interval between the two) were made at 3, 7, 20 or 40 min after each of five successive sustained contractions at 1/3 maximum tension; the succeeding sustained contraction followed one minute after these two maximum efforts were made.

Muscle temperatures were measured by inserting a 40 s.w.g. copper-constantan thermocouple obliquely in a distal direction through the brachioradialis muscle just below the elbow, leaving the thermocouple junction at a point approximately half way between the skin and the centre of the arm. Muscle temperatures, as described in this paper, refer to this 'representative' muscle temperature.

RESULTS

Effect of water temperature and recovery interval on duration of contractions

Figure 1 shows the duration of the five successive contractions for each subject in each of the eight experimental conditions. While considerable individual variation is evident in the recorded durations of contractions in any given experimental condition, the response followed a similar general pattern for each subject. It is clear from Fig. 1 that it is necessary in all experimental conditions to consider the durations of the first contractions separately from those of subsequent contractions, since at the time of the first contractions the experimental procedure was always the same except for immersion of the arm in water at two temperatures. Consequently, it is to be expected that for a given water temperature the first contraction in all experiments would be of similar duration for each subject. In fact the similarity may be observed in Fig. 1, and serves to demonstrate the repeatability of the results. The duration of the first contraction in water at 34° C was only about 70% of that found in water at 18° C. This agrees with previous findings (Nukada, 1955; Clarke *et al.* 1958) and this comparison can be seen more clearly in Fig. 2, which shows the average results for the four subjects.

The durations of the subsequent four contractions were profoundly affected both by water temperature and by the interval allowed between the contractions. In all experiments the durations of these contractions fell from the initial value to a lower, steady level by the last two contractions and in any of the eight experimental conditions the durations of these last two contractions were not significantly different. When the interval between contractions was 20 or 40 min, the duration of all five contractions remained much greater in water at 18° C than at 34° C. But when the interval between the contractions was 7 or 3 min, the difference found between the durations of the first contractions in the two water temperatures was not maintained and the curves tended to converge (see Fig. 2) although the durations of the last two contractions in water at 18° C were always greater than those obtained in water at 34° C when the interval between the contractions was held constant at 3 or 7 min ($P < 0.001$).

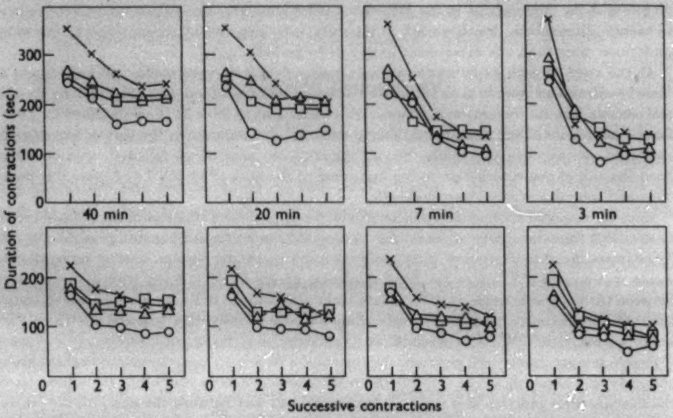

Fig. 1. Durations of five successive contractions for four individual subjects (represented by different symbols) when their arms were immersed in water at 18° C (upper diagrams) or 34° C.

In both water temperatures, the shorter the interval between the contractions, the shorter was the duration of the last two contractions. Reduction of the interval between contractions from 40 to 3 min in water at 18° C resulted in a fall in duration (mean for 4 men: see Fig. 2) from 210 to 103 sec, a fall of 107 sec. The corresponding figures in water at 34° C were 132 to 76 sec, a fall of only 56 sec, or about half that found in cool water.

Muscle temperature changes

Previous work (Clarke *et al.* 1958) showed that after a sustained contraction in water at 18 or 34° C there was a sudden rise of muscle temperature, presumably due to the post-exercise hyperaemia. This rise of temperature usually reached a peak about 5 min after the end of the contraction, and thereafter the muscle temperature fell slowly to approximate its initial level in about 20 min. This transient rise in temperature was about 1° C when the forearm was in water at 34° C, but was much greater, about 4° C, in water at 18° C, presumably owing to the much greater temperature gradient existing between the incoming blood and the tissues of the forearm in the cooler water. It remained possible that in the present experiments, when the interval between contractions was reduced to 7 or 3 min, the muscle temperature would rise progressively owing to succeeding contractions occurring during the period when the muscle temperature was raised by the post-exercise hyperaemia resulting from the preceding contractions. This rise would be expected to be greater in water at 18° C, and the changes in durations of contractions shown in Figs. 1 and 2 when the interval was 7 or 3 min might be attributable to such muscle temperature changes throughout the experiments.

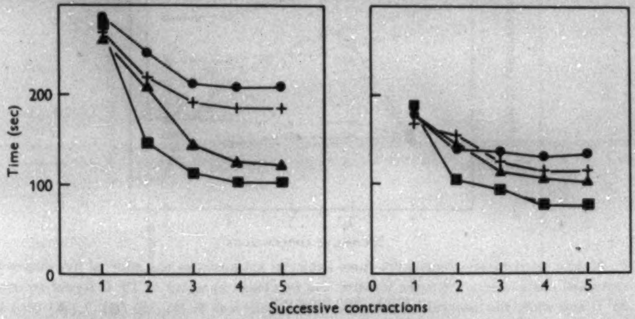

Fig. 2. The average durations of successive, sustained contractions in water at 18° C (on left) and 34° C, when the interval between contractions was 40 (●), 20 (+), 7 (▲) or 3 (■) min.

Figure 3 shows the muscle temperatures (mean of three subjects) measured at the start of each contraction in the eight experimental conditions. As postulated above, a rise of muscle temperature occurred when the interval between contractions was reduced, and a greater rise was found in water at 18° C than in water at 34° C. The temperature, however, did not rise progressively throughout the experiments when the intervals between contractions were 3 or 7 min; the rise occurred after the first two or three contractions, and thereafter the temperature remained steady or fell slightly to a steady level for the last two contractions. This is presumably owing to smaller rises of temperature following progressively shorter contractions. Reduction of the interval from 40 to 3 min resulted in a rise of muscle temperature for the last two contractions of about 0·6° C in water at 34° C, but at 18° C the corresponding rise amounted to about 5° C.

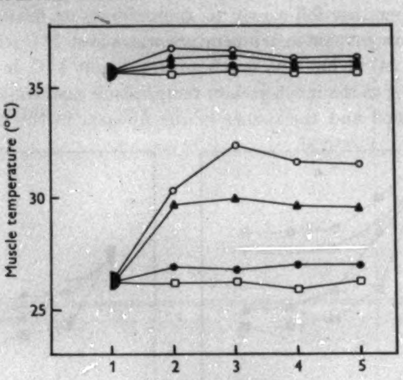

Fig. 3. Muscle temperatures (means for three subjects) measured at the start of five successive, sustained contractions when the forearm was immersed in water at 18° C (lower traces) or 34° C and when the interval between the contractions was 40 (□), 20 (●), 7 (▲) or 3 (○) min.

Recovery of muscle function

It has been noted that in any experimental condition the durations of the last two contractions were not different. If it is then assumed that this fact demonstrates the existence of a 'steady state' between the metabolic requirements of the muscle to perform sustained contractions at the required tension and the time allowed for recovery of muscle function between successive contractions, then the durations of these contractions may be considered to be a measure of the recovery of muscle function after fatigue.

In Fig. 4 the zero point on the abscissa represents the point at which a contraction ended (i.e. the point of fatigue for the maintenance of a contraction of 1/3 maximum tension) and thereafter the interval between contractions represents the 'recovery time'. Against 'recovery time' is plotted the duration of contractions in the 'steady state' (the average of the durations of the last two contractions) expressed as a percentage of the duration of the initial contractions. The points shown in Fig. 4 represent the average for four subjects for each of the eight experimental conditions.

It is evident from Fig. 4 that there were two phases in the recovery of the ability of the muscle to maintain submaximal contractions. An initial, fast rate of recovery occurred within about the first 10 min after fatigue, when more than 50% of the total recovery occurred. This was followed by a slower rate of recovery, during 30 min of which only a further 20–25% of the total recovery was achieved. After 40 min the muscle was able to maintain contractions at the required tension for only 70–75% of the initial maximum duration at either water temperature. The rate of recovery in the 7 min period following fatigue was found to be just significantly faster in water at 34° C than in water at 18° C ($P < 0\cdot5$). There was no difference due to water temperature in the rate of recovery in the period from 7 to 40 min after fatigue. If this second, slower rate of recovery did not become further slowed, then extrapolation suggests that an interval of more than 90 min would be necessary for complete recovery.

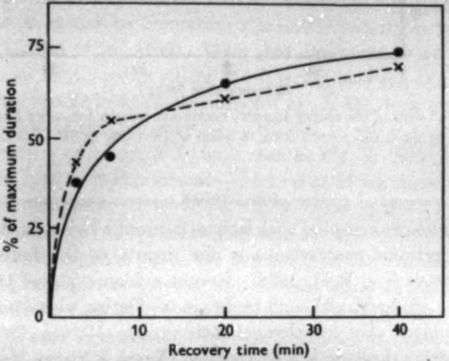

Fig. 4. The duration of sustained contractions after different lengths of time allowed for recovery from fatigue in water at 18° C (●) or 34° C (×).

For the second series of experiments where the greatest tension that could be exerted was determined at various intervals after the muscle had been fatigued by sustained contractions at 1/3 maximum tension, the tension that could be exerted has been plotted (Fig. 5) as a percentage of the initial maximum value, against the time allowed for recovery following fatigue. In these experiments the point of fatigue represents the moment when the maximum tension that can be exerted had fallen to 1/3 maximum. It is clear from Fig. 5 that the recovery of the ability to exert maximum tension is complete within 20 min after fatigue, and after 7 min is more than 90% complete.

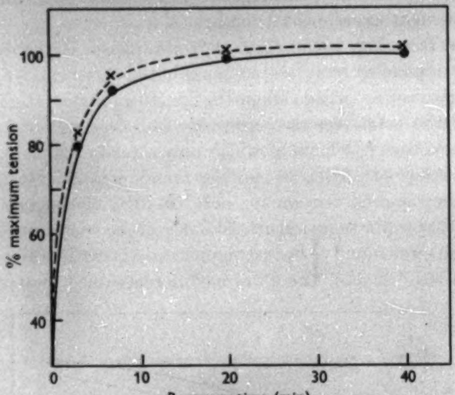

Fig. 5. The recovery of the ability to exert maximum tension following fatigue induced by sustained contractions, in water at 18° C (●) or 34° C (×).

DISCUSSION

The view is no longer accepted that fatigue induced by sustained contractions or bouts of rhythmic contractions is due mainly to the failure of central nervous pathways (e.g. Reid, 1928). Recent evidence places the site of the fatigue at the periphery, although there are conflicting views on whether the fatigue is due only to chemical changes in the muscle (Merton, 1954) or whether the neuromuscular junction is also involved (Naess & Storm-Mathisen, 1955; Krnjevic & Miledi, 1958). The belief that the neuromuscular junction may be responsible, in part at least, for fatigue induced by muscular contractions results mainly from observations made during experiments when maximum tensions were being exerted. It is doubtful whether in submaximal, sustained contractions of the type investigated here, the neuromuscular junction would be so affected. It has been shown that the frequency of stimulation necessary to elicit maximum tension in healthy human muscle is usually of the order of 35–40/sec (e.g. Bigland & Lippold, 1954) although frequencies as high as 60/sec have been recorded (Seyffarth, 1940). Also, tension has been shown to be almost linearly related to the frequency of stimulation (Bigland & Lippold, 1954) and a tension of 1/3 maximum would be associated with frequencies probably of much less than 20/sec. Smith (1934) observed frequencies of 5–12/sec during sustained contractions of unspecified strength. At such low frequencies no failure of the neuromuscular junction has been demonstrated (e.g. Brown & Burns, 1949; Krnjevic & Miledi, 1958). The balance of evidence suggests that neuromuscular failure is probably not responsible for the muscular fatigue induced in the present experiments, where the tension was maintained at 1/3 maximum tension and, in the following discussion, the assumption is made that the muscular fatigue here is due only to chemical changes occurring within the muscle.

The results show that the durations of five successive, sustained, submaximal contractions held to the point when the required tension could no longer be maintained, fall from the initial value to a lower, steady duration which is dependent partly on the temperature of the muscle and partly on the amount of time allowed for recovery between the contractions. When the interval between the contractions is kept constant at a given value, the durations of these final contractions are always longer when the forearm is immersed in water at 18° C than in water at 34° C. The reason for this is probably to be found in the faster rate of accumulation of metabolites with increasing muscle temperature (Clarke *et al.* 1958). When the temperature of the water is kept constant at 18° C, reduction of the interval between the contractions from 40 to 3 min results in a fall, by some 107 sec, in the durations of the final contractions. Part of this fall may be attributed to the concomitant rise of muscle temperature of about 5° C, as is seen in Fig. 3. Evidence from the present and from previous experiments (Clarke *et al.* 1958), shows that over the range of muscle temperatures involved, the decrease in the duration of sustained contractions (held at 1/3 maximum tension) due only to the change of muscle temperature is about 10 sec per degree C. Of the decrease in duration of 107 sec, about 50 sec is estimated to be due to the rise of muscle temperature, so that the fall due only to the reduction in the interval between contractions must be about 57 sec. In water at 34° C, the corresponding fall in duration was 56 sec, of which about 6 sec is attributable to the rise of muscle temperature, and about 50 sec is due to the reduction of the interval between contractions. The decrease in durations of final contractions estimated in this way to be due only to the reduction of the time allowed for the muscle to recover is in reasonable agreement at both water temperatures. The difference may be due to small differences in siting the thermocouple in the muscle or to differences observed in the rates of recovery (see Fig. 4) in the two water-baths.

It is surprising to find that, after contractions lasting only some 1·5–5 min, even 40 min after fatigue the durations of sustained contractions are only 70–75% of the initial value. Examination of the curves representing the recovery of the ability of the muscle to maintain submaximal contractions shows that there is a fast rate of recovery during the first 10 min after fatigue, followed by a much slower rate of recovery which is not complete within 40 min. Concurrent with the completion of the fast rate of recovery are two events: (1) the full recovery of the ability to exert maximum tension, and (2) the completion of the post-contraction hyperaemia (Clarke *et al.* 1958). Thus the second, slow rate of recovery is not associated with hyperaemia, and occurs after the muscle has recovered its ability to exert maximum tension. These facts suggest that two different processes may occur in the recovery of the ability of the muscle to sustain submaximal contractions, the first probably involving the removal of metabolites from the muscle during the post-contraction hyperaemia while the second process may well involve the slow replacement in the muscle of reserves of materials necessary for contraction, which are depleted by the sustained contractions. Such a concept would also be compatible with the gradual decrease in duration of successive sustained contractions, possibly by a cumulative depletion of reserves of materials necessary for contraction in the muscle.

In considering the events occurring in the muscle immediately after fatigue, an economical hypothesis worth considering is that the substance which accumulates in the muscle to cause fatigue is the same as that which causes the post-exercise hyperaemia. If the arguments above are accepted, the incomplete recovery of the ability of the muscle to sustain contractions at the end of the period of hyperaemia does not invalidate such a hypothesis. Dornhorst & Whelan (1953) and Patterson & Shepherd (1954) considered that the substance causing the post-exercise hyperaemia is removed from the muscle at a rate independent of the rate of blood flow, provided that this is above 'some low level'. In the present experiments, the rate of recovery of muscle function is just significantly faster in the warmer water during the hyperaemia period, and this might be taken, at first sight, to show that the substances causing the fatigue and the hyperaemia are not identical. This phenomenon, however, may be due to the faster replacement of reserves of materials to the muscle during this period, or to the expected faster rate of chemical reactions due solely to the higher muscle temperature (Abramson, Kahn, Tuck, Turman, Rejal & Fleischer, 1957), or to a combination of these two factors. While there is no direct evidence to support the hypothesis that the substance causing fatigue also causes the post-exercise hyperaemia, there is no evidence on which to reject it.

SUMMARY

1. Five successive, sustained contractions at a tension of 1/3 maximum tension were held by four subjects when the forearm was immersed in water at 18° C or at 34° C. The intervals between the contractions were 3, 7, 20 or 40 min.

2. The duration of contractions for a given interval between contractions was always greater in water at 18° C than in water at 34° C.

3. The duration of the final contractions fell markedly at both water temperatures when the interval between contractions was reduced from 40 to 3 min. In water at 18° C this fall was nearly twice as great as in water at 34° C; this was attributable to a greater concomitant rise in muscle tempera-

ture during the experiments, due to the reduction of the interval between contractions, when the arm was in water at 18° C than when in water at 34° C.

4. The recovery of the ability to sustain submaximal contractions after fatigue was only 70–75% complete 40 min after fatigue. Recovery occurred at a fast rate immediately after the contraction, and at a much slower rate later. Recovery of the ability to exert maximum tension was completed in 10 min.

I wish to express my gratitude to D. E. Lee and E. S. Reeves for invaluable technical help and for their co-operation as subjects in these experiments.

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EFFECT OF ABLATION OF MEDULLARY EMETIC CHEMORECEPTOR TRIGGER ZONE ON VOMITING RESPONSES TO CEREBRAL INTRAVENTRICULAR INJECTION OF ADRENALINE, APOMORPHINE AND PILOCARPINE IN THE CAT

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Vomiting is a frequent response to drugs injected into the cerebral ventricles in cat, dog and man (Cushing, 1931; Feldberg & Sherwood, 1954; Weinberg & Haley, 1955). Ablation of the emetic chemoreceptor trigger zone in the medulla oblongata abolishes vomiting in response to certain drugs given by systemic routes of administration in dog and cat (Borison & Wang, 1953).

The experiments reported here show that in cats ablation of the chemoreceptor trigger zone abolishes the emetic response to adrenaline and apomorphine injected into the cerebral ventricles and reduces the incidence of vomiting in response to intraventricular pilocarpine. A preliminary account of the results has been given to the Physiological Society (Borison, 1958).

METHODS

Cats were prepared with cannulae permanently implanted in the lateral cerebral ventricle by means of the technique described by Feldberg & Sherwood (1954). Drugs were injected intraventricularly in a volume of 0·25 ml.; the injections were spaced at approximately weekly intervals. Between drug tests, the cannulae were washed routinely with an injection of 0·25 ml. NaCl solution 0·9% (w/v); no untoward effects were elicited by the saline injection. The drugs were administered as hydrochloride salts. The doses of apomorphine and pilocarpine refer to the salts; those of adrenaline to the base. The drugs were dissolved in NaCl solution 0·9% (w/v) or occasionally in Locke's solution, except for the largest doses of apomorphine which had to be dissolved in distilled water. Intraventricular injection of 0·25 ml. of distilled water evoked no effects different from those obtained after injection of physiological saline solution.

Lesions of the medullary emetic chemoreceptor trigger zone were made under pentobarbitone anaesthesia by gentle thermal cauterization of the lateral portion of the area postrema. Desacetyl-lanatoside C (Cedilanid, Sandoz) was given intravenously in the dose of 0·16 mg/kg as a functional test for the adequacy of the ablation procedure; failure to elicit vomiting was taken as evidence of successful ablation (Borison, 1952). Emetic tests were performed shortly after the cats had been fed and only the actual expulsion of vomitus was considered to be a positive emetic response.

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Occasionally an animal failed to give the characteristic emetic response to intraventricular drug injection and responded instead with some unusual effect, such as a falling reaction or other convulsive disturbance. In such cases the cannula was found either to be loose or to be plugged, and recannulation at another site usually restored the original sensitivity to emetics. The placing of the cannula in the lateral ventricle was confirmed at post-mortem examination of the brain after perfusion with formalin.

RESULTS

Adrenaline. The dose of 0·15 mg of intraventricular adrenaline evoked vomiting in fourteen out of sixteen control cats. The two cats which failed to vomit were found, at post-mortem examination, to have cerebral abscesses surrounding the tips of the cannulae. Hence 0·15 mg can be considered a uniformly effective emetic dose in normal cats. Vomiting generally occurred within 10 min after intraventricular injection of adrenaline and the average latent period in this series of experiments was 4·5 min. Intravenous injection of 0·15 mg of adrenaline in two cats did not cause vomiting. Repeated weekly tests with intraventricular adrenaline for periods as long as one month did not cause development of tolerance as judged by the fact that consistent responses were obtained without prolongation in the latency of emesis.

By contrast, not a single emetic response was evoked in trigger-zone-ablated cats with doses up to 0·5 mg. Five of the medullary-lesion cats had been tested pre-operatively with 0·15 mg intraventricular adrenaline and all of them had given normal vomiting responses.

In addition to the vomiting, intraventricular adrenaline in control cats evoked certain behavioural effects, such as apparent lethargy and postural plasticity, as described in detail by Feldberg & Sherwood (1954). These effects were especially evident at the higher dose levels and they invariably followed the onset of vomiting. Trigger-zone ablation which selectively abolished the emetic response did not modify the general behavioural effects to intraventricular adrenaline.

Apomorphine. In control cats emetic responses occurred regularly after intraventricular injections of 0·1–1 mg apomorphine. The responses occurred more promptly than after adrenaline. The latency for emesis was usually between 2·0 and 2·5 min, but one cat vomited as early as 1 min after 0·5 mg, and one cat vomited as late as 4 min after 0·05 mg.

Chemoreceptor-trigger-zone ablation eliminated the vomiting response to intraventricular apomorphine given in doses from 0·25 to 0·5 mg. Two of the medullary-lesion animals had been tested pre-operatively with intraventricular apomorphine and both had vomited in response to 0·05 mg. Of four trigger-zone-ablated cats tested with 1·0 mg apomorphine three did not vomit and one vomited after a latent period of 3 min; the same cat failed to vomit in response to 0·5 mg apomorphine.

In the control cats vomiting was the only observable response to 0·1 mg intraventricular apomorphine. With larger doses the cats became increasingly excitable to sound and touch, and they displayed dilated pupils and defensive movements which are the characteristic reactions of this species when apomorphine is given systemically. The striking difference between systemic and intraventricular apomorphine, however, is the effectiveness of intraventricular injection in evoking emesis in the absence of general behavioural effects. Vomiting after systemic apomorphine can be evoked only with doses of the order of 25 mg/kg and never in the absence of the behavioural excitatory effects. Trigger-zone ablation eliminated the vomiting response to intraventricular apomorphine without detectably altering the general behavioural reactions to the drug.

Pilocarpine. An intraventricular injection of 1 mg pilocarpine evoked vomiting in five out of six control cats; the latency varied from 3 to 11 min, with an average of 6 min. The same dose evoked vomiting in one out of four chemoreceptor-trigger-zone ablated cats after a latency of 10 min. At the dose level of 2·0 mg vomiting was evoked in two out of five operated cats after latencies of 5 and 17 min, respectively. Cats which vomited after 1·0 and 2·0 mg pilocarpine responded a second time when tested again with the same doses; these cats were not tested at higher dose levels. Three remaining trigger-zone-ablated cats, which did not vomit in response to 2·0 mg, were refractory as well to 3·0 mg. One of these cats was tested also with 4·0 mg and still did not vomit. Two of the medullary-lesion animals had been tested pre-operatively with 1·0 mg intraventricular pilocarpine; one had vomited and one had not. Post-operatively both were refractory to 3·0 mg.

In addition to the vomiting, intraventricular pilocarpine evoked, in control cats, pronounced salivation, panting, head scratching, tremor and occasionally a rage reaction which included pilo-erection, pupillary dilatation, defensive posture and growling. Trigger-zone ablation, when effective, prevented only the emetic response. Occasionally a single unproductive retch occurred within a few seconds after injection in normal as well as in operated cats. This effect bore no consistent relationship to the subsequent occurrence of vomiting. Atropine (0·5 mg/kg) injected intraperitoneally terminated the whole behavioural reaction, which otherwise lasted for hours.

Sham medullary operation. Four cats were subjected to an operative procedure identical with that employed for ablation of the medullary emetic chemoreceptor-trigger zone, except that cauterization of the area postrema was not performed. Vomiting was evoked in these animals, with both intra ventricular adrenaline and apomorphine, but it was necessary in two cats to use larger doses (0·25 mg) than were required in control unoperated animals. Nevertheless, the doses of adrenaline and apomorphine which were effective in causing vomiting in sham-operated cats were consistently without emetic effect in trigger-zone-ablated cats.

Oral copper sulphate. A gastro-intestinal irritant was administered to trigger-zone-ablated cats as a motor function test of the emetic apparatus. Copper sulphate was given by stomach tube after food had been withheld from the cats for 18 hr. The dose of 25 ml. of $CuSO4.5H_2O$ in aqueous solution (125 mg/100 ml.) caused vomiting in three out of six operated cats within 1 hr after administration. One of the three remaining cats vomited when the concentration was increased to 250 mg/100 ml. These results show that trigger-zone-ablation abolishes the emetic reactivity to intraventricular drugs without impairing the motor function of vomiting.

Distribution of intraventricular dye. Evans Blue dye, 0·25 ml. of a 1·0% concentration in Locke's solution, was injected intraventricularly in the trigger-zone-ablated cats after they had been anaesthetized with pentobarbitone; 10–30 min after injection of the dye they were perfused with formalin. No influence on dye distribution could be attributed either to the time interval between injection and perfusion, or to the chronicity of the medullary operative procedure. The general pattern of dye distribution was as follows: the entire ventricular system was filled symmetrically; all subarachnoid surfaces of the lower brain stem, except for the dorsum of the cerebellum, were stained intensely; the dye extended rostrally to the peri-infundibular region and it also reached the pre-amygdaloid area but with less intensity; the dorsal surfaces of the cerebral hemispheres were not stained. In two cats the fourth ventricle was only lightly stained in the region of the obex, but the cisterna was coloured intensely blue. In one cat the ala cinerea on one side was not stained, probably owing to post-operative adhesion to the cerebellum.

Attempts were made in three cats to cannulate the fourth ventricle by the dorsal approach through the cerebellum. At post-mortem examination it was found that the cannulae extended to the base of the cerebellum, but the polythene tips had failed to puncture the roof of the fourth ventricle. In one cat injection of dye solution through the cannula intensely stained the subarachnoidal surfaces of the cerebellum and lower brain stem, while the ventricular system was not stained at all. Although the fourth ventricle had not been cannulated in these cats, injection of 0·25 mg of either adrenaline or apomorphine evoked vomiting after latent periods of the same order of duration as after injection into the lateral cerebral ventricle, but the usual behavioural reactions obtained by lateral intraventricular injection were absent.

DISCUSSION

The abolition of vomiting in response to intraventricular adrenaline or apomorphine, as a result of ablating the medullary emetic-chemoreceptor-trigger zone, suggests that they either act directly on the trigger zone after passing through the ventricular system, or that they act more rostrally in a cerebral ventricle, the excitatory effect then being transmitted to the lower brain stem through a descending pathway which passes in the immediate vicinity of the trigger zone. In support of a direct local action at the trigger zone are the findings that on intraventricular injection of 0·25 ml. of a solution of dye the dye reached the lower brain stem by displacement of cerebrospinal fluid, and that adrenaline also exerted its emetic effect on lower-brain-stem subarachnoidal injection. Further, Hatcher & Weiss (1923) evoked vomiting by direct application of adrenaline and apomorphine on the medulla oblongata in the dog. However, the fact that there is a species difference between dog and cat in emetic sensitivity to drugs weakens the argument based on local application, and thus the possibility of a more rostral action has not been fully excluded.

Adrenaline is not known to cause emesis after systemic administration and, indeed, 150 μg intravenously failed to evoke vomiting in the cat. However, nausea and vomiting are symptoms associated with phaeochromocytoma, a tumour of the adrenal medulla in man which causes hypersecretion of adrenaline. The latency for vomiting after intraventricular adrenaline can be considered longer than the time required for the drug to reach the lower brain stem. It would appear, therefore, that the latent period for emesis represents largely the time for activation of the emetic receptors.

The potency and specificity of emetic effect of intraventricular apomorphine were not expected, because the cat, unlike the dog, is insensitive to the drug when it is given systemically. The question arises, then, whether the species difference in emetic reactivity of cats and dogs to systemic apomorphine depends on a difference in blood–brain barrier drug selectivity. Such a mechanism would be consistent with the present findings.

Certain differences are evident in the emetic action of intraventricular pilocarpine as compared to adrenaline and apomorphine. Doses of pilocarpine required to evoke vomiting by intraventricular injection were of the same order of magnitude as are needed by intravenous injection and, accordingly, severe and persistent side effects resulted. Trigger-zone ablation did not regularly abolish the vomiting response to intraventricular pilocarpine. This observation is in agreement with previous findings that in dogs local application of pilocarpine on the medulla oblongata did not evoke emesis (Hatcher & Weiss, 1923), and that in cats the emetic response to intravenous pilocarpine persisted after ablation of the trigger zone and could be localized in the basal cortex of the frontal lobe (Borison, Harris & Schmidt, 1956). If we assume that it is necessary for intraventricular pilocarpine to reach this site in order to evoke emesis, the low concentration of dye found at the base of the frontal lobe after intraventricular injection could explain the high dosage required for the drug as a consequence of dilution in the cerebrospinal fluid. Fortuitous interruption of corticobulbar pathways to the emetic centre, as a result of ablating the trigger zone, could account for the reduction in the incidence of vomiting in response to intraventricular pilocarpine.

SUMMARY

1. Emetic doses were determined for adrenaline, apomorphine and pilocarpine administered by cerebral intraventricular injection through permanently implanted cannulae in normal cats.

2. Chronic ablation of the emetic chemoreceptor trigger zone, in the area postrema of the medulla oblongata, abolished the vomiting responses to intraventricular adrenaline and apomorphine, and reduced the incidence of vomiting induced by intraventricular pilocarpine. Behavioural effects of the drugs remained unaltered.

3. Vomiting was evoked in trigger-zone-ablated cats by oral copper sulphate, thus demonstrating that the medullary lesion conferred selective emetic refractoriness to intraventricular adrenaline and apomorphine without impairing the motor function of vomiting.

4. Evans Blue dye injected intraventricularly in trigger-zone-ablated cats in the same volume as the drugs stained the entire ventricular system as well as the subarachnoidal surface of the lower brain stem. This finding is consistent with the hypothesis of a direct emetic action of intraventricular adrenaline and apomorphine at the medullary emetic chemoreceptor trigger zone.

The author wishes to thank Sir Charles Harington for hospitality and Dr Feldberg for his interest and encouragement.

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A STUDY OF SUPERSENSITIVITY IN DENERVATED MAMMALIAN SKELETAL MUSCLE

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It is well known that organs chronically deprived of their motor nerves develop an increased sensitivity to the neurohumoral transmitter and to other chemical agents. This phenomenon is observed in several types of tissue, e.g. striated muscle, smooth muscle, ganglia and glands, and is known as 'denervation supersensitivity' (Cannon & Rosenblueth, 1949). The mechanism underlying the change has been extensively studied and has been the subject of much speculation, but it has, up to now, remained unknown (Emmelin, 1952).

Supersensitivity is particularly marked in chronically denervated skeletal muscle. Denervated frog muscle is overexcitable to acetylcholine (ACh) by a factor of about one hundred (Nicholls, 1956), and denervated mammalian muscle (cat gastrocnemius) becomes one thousand times more excitable to ACh administered by close arterial injection (Brown, 1937).

The sensitivity in striated muscle to ACh and other chemicals is increased far more than that to electric stimulation, and cannot be explained by changes in the electric properties of the muscle membrane (Nicholls, 1956). It is consequently reasonable to suppose that supersensitivity following denervation results from an increase in the chemical excitability of the muscle, with an enhanced responsiveness of the membrane to the depolarizing action of ACh.

Recent electrical methods for close-range, localized drug application (Nastuk, 1953) are particularly suited for studying drug effects on small and limited parts of the muscle membrane (del Castillo & Katz, 1955*a*). Iontophoretic micro-application of ACh to single muscle fibres is for that reason likely to offer a possibility for investigating the distribution and sensitivity of ACh receptors in denervated muscle. In the present study we have used the technique of localized ACh application with the chronically denervated tenuissimus muscle of the cat. It will be shown that after denervation the entire muscle membrane becomes as sensitive to ACh as the end-plate region, which maintains its original responsiveness to the drug. A preliminary report of some of the observations has been given (Axelsson & Thesleff, 1959).

METHODS

Unless otherwise stated, the experiments were made on the isolated tenuissimus muscle of the cat. This muscle has the advantage of being covered by only a thin layer of connective tissue and can be kept for a long time in oxygenated Tyrode fluid without showing signs of deterioration (Boyd & Martin, 1956*a*); for details of its anatomy see Adrian (1925).

For denervation and removal of the muscle the cats were anaesthetized with Nembutal (pentobarbitone; Abbott Laboratories) 40 mg/kg body wt., injected intraperitoneally. Denervation was carried out 2–21 days before the experiment by the removal of 1 cm of the motor nerve close to its point of entry into the muscle. Generally only the muscle on one side was denervated, while the other served as control. Denervated and control muscles were always examined on the same day.

During the experiment the muscle was mounted around a Perspex rod in a constant-temperature bath kept at 35–37° C. The bathing fluid had the composition used by Liley (1956), and it was oxygenated by bubbling a O_2 95% + CO_2 5% mixture through it immediately before its introduction into the muscle bath. The bath held about 30 ml. of solution, which was changed continuously at the rate of about 500 ml./hr.

The drugs used in the study were released iontophoretically from the tip of a micro-pipette as described by del Castillo & Katz (1955*a*). Drug pipettes had a tip diameter of less than $0{\cdot}5\,\mu$, and the discharge of the drug was regulated by 'breaking' or 'releasing' voltages (making the interior of the pipette more negative, or positive, respectively). The sensitivity of the muscle membrane to ACh was tested by applying positive voltage pulses of constant intensity and duration (1–10 msec) to the pipette. When the tip of the pipette was close to the receptor structure, the ACh released by a current pulse produced a transient membrane depolarization of a few millivolts amplitude. The position of the tip of the pipette was then adjusted, until the ACh potential reached its shortest rising time and highest peak amplitude. In an innervated muscle the end-plate region was located by pursuing fine superficial nerve twigs and by the recording of miniature end-plate potentials with a rapid time course.

The resting membrane potential and membrane potential changes were recorded with conventional capillary micro-electrodes, as described by Fatt & Katz (1951). The tip of the micro-electrode was inserted into the muscle fibre within 0·1 mm of the point of drug application. The time constant with a 10 MΩ electrode was of the order of 50 μsec. The current passing through the drug pipette was in all instances monitored and recorded on the second beam of the oscilloscope.

The drug pipette and the micro-electrode were carried on a Zeiss slide micromanipulator and their position was viewed with a binocular dissecting microscope at 80 × magnification.

The drugs employed in the study were: acetylcholine and edrophonium chloride (Hoffmann-La Roche), carbachol and tubocurarine chloride (Burroughs Wellcome).

RESULTS

Sensitivity to ACh

In an innervated muscle ACh produces membrane depolarization only when applied to the end-plate region of a muscle fibre (Figs. 1 and 2). The area sensitive to ACh is small and covers not more than 0·1 mm of the fibre length. Within this limited part of the membrane an ACh pulse of 1–10 msec duration produces a transient membrane depolarization, which at critical 'spots' may have a rising phase lasting about 10 msec, as is shown in the lower record of Fig. 3. At such a 'spot', however, the position of the pipette is critical and small movements horizontally or vertically cause the potential to decline to a small fraction and the rising phase to lengthen several times. In a muscle with intact innervation it is not possible by microapplication of ACh to produce membrane depolarization outside the end-plate area (Figs. 1 and 2).

In chronically denervated muscles, however, the entire muscle membrane is sensitive to applied ACh (Fig. 1). Four to five days after denervation, locally released ACh produces depolarization with a slow time course, outside the former end-plate region. After one week of denervation and subsequently throughout our observation period (21 days) the whole surface of the muscle is

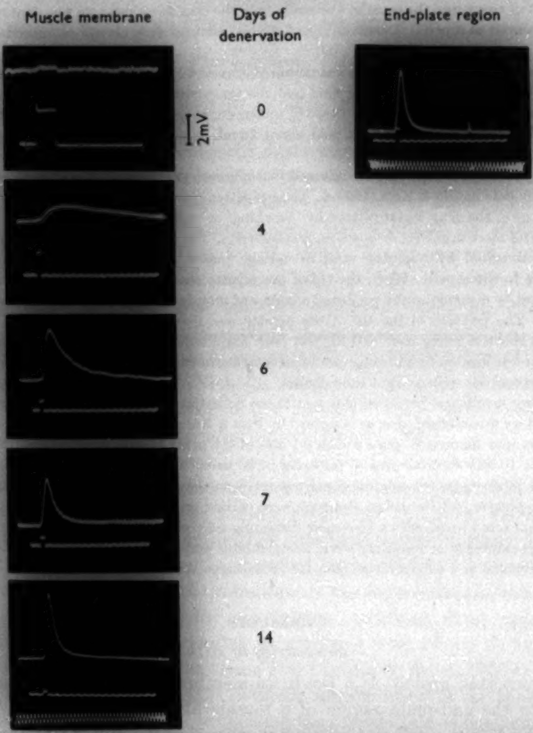

Fig. 1. An illustration of the sensitivity of innervated and denervated muscle fibres to iontophoretically applied ACh. The membrane potential of the fibre is recorded in the upper tracing and the current passing through the pipette in the lower tracing of each record. In an innervated muscle ACh produces membrane depolarization only when applied to the end-plate region (upper right record) but has no effect when released outside this area of the membrane (upper left record). After denervation the whole muscle becomes sensitive to ACh, which wherever applied to the membrane causes a potential change (subsequent records). Time marker, 100 c/s.

uniformly sensitive to ACh. As an example of this, it can be mentioned that in twelve muscles, denervated 1–3 weeks previously, there was among more than two hundred tested spots not a single one that was insensitive to applied ACh. The uniformity of ACh sensitivity in denervated muscles made only vertical movements of the pipette critical for the amplitude and time course of an ACh potential. This is illustrated in Fig. 2, where the sensitivity of a muscle fibre to ACh was determined by moving the tip of the pipette close to the fibre membrane at points separated by distances of 0·5 mm. In a 14-days denervated muscle (upper record) an ACh pulse of constant intensity and duration produced a membrane depolarization of about similar amplitude and time course each time the pipette approached the membrane, while in an innervated fibre (lower record) ACh was effective only when applied at a visible end-plate region. Because of damage caused to the muscle membrane by the insertion of the recording electrode, only about six points were tested in each muscle fibre.

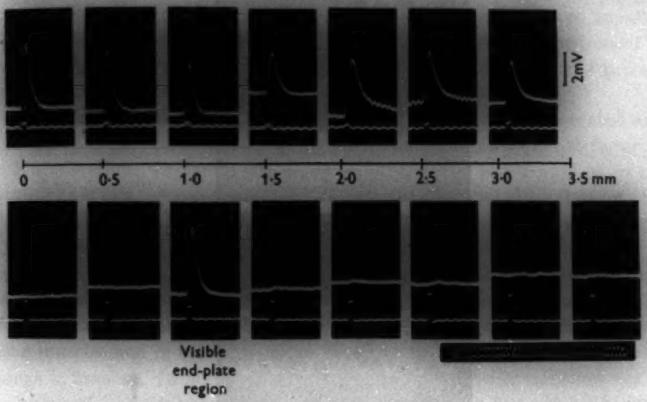

Fig. 2. The sensitivity of a muscle fibre to locally applied ACh was tested by moving the tip of an ACh pipette close to the muscle membrane at points separated by distances of about 0·5 mm. In an innervated fibre (lower records) only the visible end-plate region is sensitive to ACh. In a 14-days denervated fibre a constant pulse of ACh produced at each point of the membrane a potential change, as is shown in upper records. To obtain a maximum response only vertical movements of the drug pipette were used. Time marker, 100 c/s.

The time courses of an ACh potential at a sensitive 'spot' in an innervated end-plate region and that observed anywhere in a 2-weeks denervated muscle were similar, as is shown in Fig. 3. Time to peak and amplitude of an ACh potential are related to the distance between the tip of the pipette and the receptor site in a way depending on the principles of diffusion (del Castillo &

Katz, 1955*a*). The formula given by del Castillo & Katz suggests that for ACh potentials with a rising phase of 10 msec duration (Fig. 3), the distance between source and receptor is about 10 μ, and that consequently the spacing of receptors in a denervated muscle membrane corresponds to that of an innervated end-plate. Another method of estimating the 'sensitivity' of ACh receptors is to calculate the number of coulombs responsible for the release of ACh ions from the tip of the micro-pipette (del Castillo & Katz, 1957). This was done in experiments such as those shown in Fig. 1, and the results are given in Table 1, expressed as C/mV amplitude of the resulting ACh potential. The table shows that the quantity of ACh^+ which produces a given depolarization in a denervated muscle is of the same order of magnitude

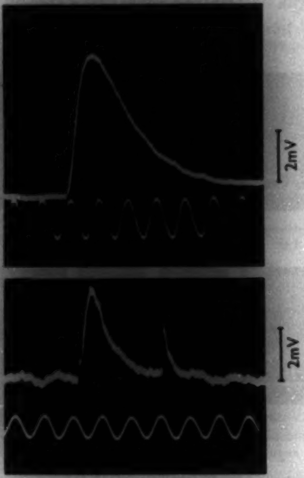

Fig. 3. The time course of an ACh potential produced by a current pulse of 1 msec duration at a sensitive 'spot' in an innervated end-plate (lower record) and anywhere at the membrane of a 14-days denervated muscle fibre (upper record). Note a miniature end-plate potential after the ACh pulse in the lower record. Time marker, 100 c/s.

TABLE 1. The sensitivity of ACh receptors was estimated by calculating the numbers of coulombs required for the release of the quantity of ACh^+ that produced a membrane depolarization of one mV amplitude. The experiments were made on tenuissimus muscles after 7 and 14 days denervation and on the innervated end-plates of corresponding control muscles. The same batch of micropipettes was used in all tests. S.E.'s of the means are shown

Days of denervation	No. of fibres	C/mV
0 (end-plate region)	29	$1{\cdot}4\ (\pm 0{\cdot}13) \times 10^{-10}$
7	16	$1{\cdot}7\ (\pm 0{\cdot}24) \times 10^{-10}$
14	9	$6{\cdot}6\ (\pm 0{\cdot}73) \times 10^{-11}$

as that which causes a similar potential change at the end-plate of an innervated fibre.

A few days after denervation miniature end-plate potentials disappear and it is impossible to locate the former end-plate with certainty. However, one may observe degenerating nerve twigs and thereby obtain an idea about the approximate site of this region. In these instances we found that the supposed end-plate had a sensitivity to ACh which corresponded to that of the end-plate region in an innervated fibre. In muscles denervated for 7–21 days no particular region of the membrane was more sensitive to ACh than other parts. Consequently, it is unlikely that the end-plate, after denervation, becomes supersensitive to ACh. Presumably it retains its original responsiveness to ACh throughout the denervation period and is not affected by the process which renders the rest of the cell membrane sensitive to the drug.

Determinations have also been made of the sensitivity of innervated and chronically denervated tenuissimus muscles to more conventional forms of drug application. In these experiments the drug was either applied to the bathing fluid in a constant-temperature bath or administered by close arterial injection. To avoid errors caused by enzymotic and spontaneous hydrolyses of ACh, carbachol was used. The threshold was taken as the concentration or dose of the salt which produced a visible muscle twitch or in denervated muscles an isotonic contracture. With bath application, carbachol in an effective concentration produced an electrically silent contracture in denervated muscles and in innervated muscles fibrillary muscle twitches without a change in tension. In innervated muscles the threshold concentration was of the order of 10^{-5} (w/v), and in muscles denervated for 2 weeks 10^{-6} (w/v). Close arterial injections were made in a volume of 0·2 ml. and corresponding threshold doses were 5 and 0·05 μg.

In addition to the tenuissimus muscle of the cat, the sensitivity of chronically denervated muscles to iontophoretically applied ACh has been studied in the hemidiaphragm and in the extensor digitorum longus muscles of the rat. The results were qualitatively similar to those obtained in the tenuissimus muscle. About 2 weeks after denervation, the entire muscle membrane, with the occasional exception of the very distal end of the muscle fibre, was sensitive to locally applied ACh. The rising phase of the ACh potentials was, however, somewhat slower in a denervated muscle of the rat than it was at the end-plate region of an innervated control muscle.

Properties of ACh receptors in denervated muscle

The length of a muscle fibre in the tenuissimus muscle of the cat is about 2 cm and its diameter 30 μ (Adrian, 1925; Boyd, 1956). Assuming that the ACh-sensitive region at the end-plate covers 0·1 mm of the fibre length, it is obvious that after denervation the size of the receptor area increases by a factor of at least 200. The enormous increase of the receptor area made it desirable to ascertain whether these new ACh receptors have properties identical with those at the end-plate.

At an innervated end-plate ACh raises the Na and K conductance of the membrane and possibly also its permeability to other ions. This general permeability increase tends to push the membrane potential, irrespective of its initial level, towards an equilibrium value of about 10–20 mV, negative inside (cf. del Castillo & Katz, 1954). We have determined the equilibrium potential during the action of ACh in a 10-days-denervated muscle and established that it is similar to that at a normal end-plate. This is illustrated in Fig. 4, which shows a muscle fibre in which the membrane potential was progressively altered by passing rectangular current pulses through the membrane. (Mechanical responses were reduced by the use of a bathing fluid containing a 2 × normal concentration of NaCl, as described by Howarth, (1958). The reversal of the ACh potential occurred at a membrane potential level of about −10 mV, which is about the same equilibrium potential as that observed at an innervated end-plate.)

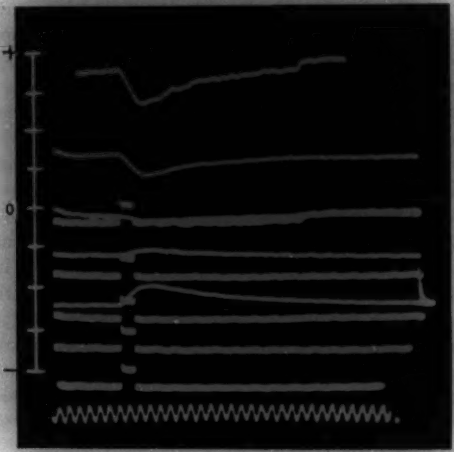

Fig. 4. The equilibrium potential during the action of ACh in a 10-days denervated muscle fibre The membrane potential of the fibre was progressively altered by passing rectangular current pulses through the membrane. At a membrane potential level of about −10 mV the direction of an ACh pulse reversed. Mechanical movements were reduced by the use of Tyrode solution with a 2 × normal concentration of NaCl. Time marker, 100 c/s; voltage calibration 10 mV steps.

As in the end-plate (del Castillo & Katz, 1955*b*), so also in the denervated muscle membrane, ACh raises the permeability to K^+ in the absence of external Na^+. This is shown in an experiment in which a denervated muscle was immersed in an isotonic solution of K_2SO_4 (Fig. 5). The membrane potential was displaced from zero by the passage of a transverse current and ACh was released from the tip of a micropipette. As a sign of increased membrane permeability the pulse of ACh reduced the potential difference built up across the membrane.

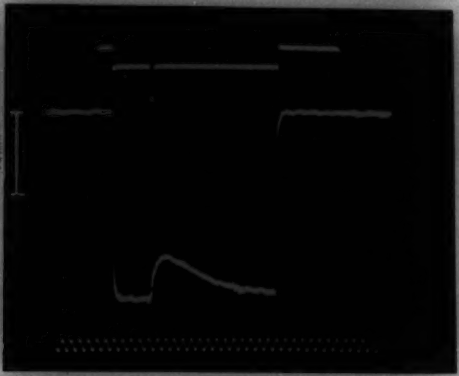

Fig. 5. ACh causes a reduction of the membrane resistance in a 7-days denervated muscle fibre immersed in isotonic K_2SO_4 solution. In this medium the cell membrane is completely depolarized but is electrotonically displaced from zero by the passing of a 'catelectrotonic' current pulse. When ACh is released its effect is to reduce the membrane resistance as is shown by the prompt decline in the electrotonic potential. Time marker, 100 c/s.

In the continued presence of ACh, end-plate receptors become refractory to the depolarizing action of the drug (Thesleff, 1955; Axelsson & Thesleff, 1958). This occurs also in a denervated muscle fibre but the time course of the 'desensitization' process is slower than at an innervated end-plate, as is shown in Fig. 6. The experiments illustrated in Fig. 6 were made with double-barrelled ACh pipettes, as described by Katz & Thesleff (1957*a*). Short 'test' pulses of ACh were released from one barrel while the other barrel was used for a prolonged and steady release of ACh. Receptor 'desensitization' is shown as a reduction in the amplitude of the test response during the continuous output of ACh.

The response of a chronically denervated muscle to some other drugs was found to be similar to that of an innervated end-plate. Carbachol released iontophoretically by a current pulse produced a transient depolarization with a rapid time course, and tubocurarine reversibly reduced the sensitivity of ACh receptors (Fig. 7).

On the other hand, with anticholinesterases a difference was found. When a drug such as edrophonium (Tensilon) is applied to the normal end-plate region it potentiates the responses to ACh, as is shown in the lower record of Fig. 8. In a denervated muscle, however, its effect is to inhibit the response to ACh and to produce a slight membrane depolarization (upper record). The general view is that the main action of edrophonium is inhibition of cholinesterase (Smith, Cohen, Pelikan & Unna, 1952; Nastuk & Alexander, 1954; Katz & Thesleff, 1957*b*); hence the absence of ACh potentiation indicates that the membrane is, in a practical sense, devoid of cholinesterase.

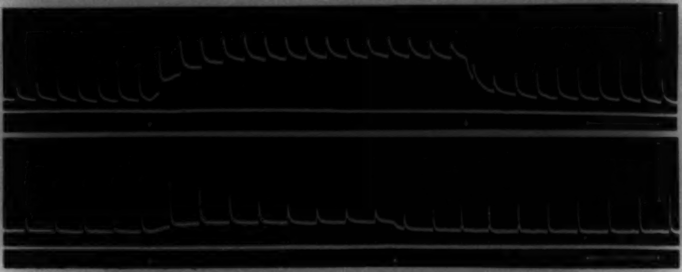

Fig. 6. 'Desensitization' of ACh receptors by the continuous release of ACh (for explanation see text). The lower record illustrates the time course of this process in an innervated end-plate region and the upper record in the membrane of a 14-days denervated muscle fibre. Time marker, 5 sec; voltage calibration 5 mV.

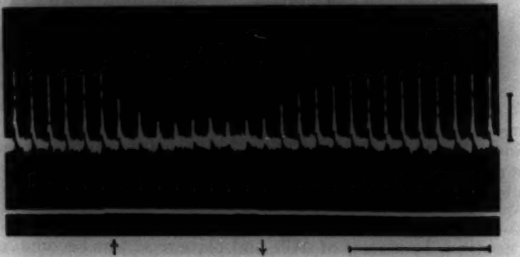

Fig. 7. Carbachol released by current pulses produces in a 14-days denervated muscle fibre transient depolarizations. When tubocurarine is released (between arrows) the sensitivity of membrane receptors is reversibly reduced, as is illustrated by the decline in amplitude of the carbachol pulses. Time marker, 5 sec; voltage calibration 2 mV.

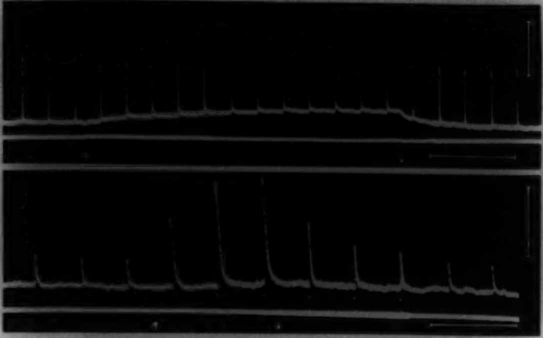

Fig. 8. The anticholinesterase drug edrophonium (Tensilon) potentiates the effect of ACh at the innervated end-plate region (lower record) and inhibits its action on the membrane of a 12-days denervated fibre (upper record). The release of edrophonium is marked by arrows. Time marker, 5 sec; voltage calibration 5 mV.

Electrical excitability

It has been suggested that, unlike the rest of the muscle membrane, the ACh receptors are not excitable by electrical stimulation (del Castillo & Katz, 1954). Grundfest (1957), moreover, has concluded that electrical inexcitability is a common feature of chemically activated post-synaptic membranes. It was therefore of some interest to examine whether a denervated muscle would, despite its high and uniform sensitivity to ACh, respond by a conducted response to electrical stimulation. This was investigated in a number of experiments in which the electrical thresholds of single fibres were determined by the use of two intracellular electrodes, one for passing a stimulating electric current and the other for recording resulting potential changes. During an observation period of 3 weeks after denervation all the fibres were electrically excitable and a depolarizing current gave rise to a propagated action potential (Fig. 9). The critical membrane potential, i.e. the potential difference across the membrane at which excitation occurred, was unaltered by denervation, as is shown in Table 2.

DISCUSSION

The present investigation has shown that in a mammalian muscle about 4 days after denervation the size of the ACh-sensitive area starts to increase and 3–6 days later covers the larger part or all of the muscle membrane. Brown (1937) observed, in cat muscle, that supersensitivity to applied ACh begins 4 days after denervation and is fully developed within 1 week. The parallelism in time course of the two processes, i.e. the increase in the receptor surface and the supersensitivity, suggests that they are closely related.

That an increase in the size of the ACh-sensitive area can produce supersensitivity to the drug is evident when the electric properties of a muscle fibre are considered. Fig. 10 is a diagram of an electric model with cable conductor properties similar to those of a muscle fibre. A potential difference (e.g. the membrane potential) between *A* and *B* can be reduced by short-circuiting either a fraction, or all, of the parallel resistors r_m. According to Ohm's law the current divides itself in inverse proportion to the relative

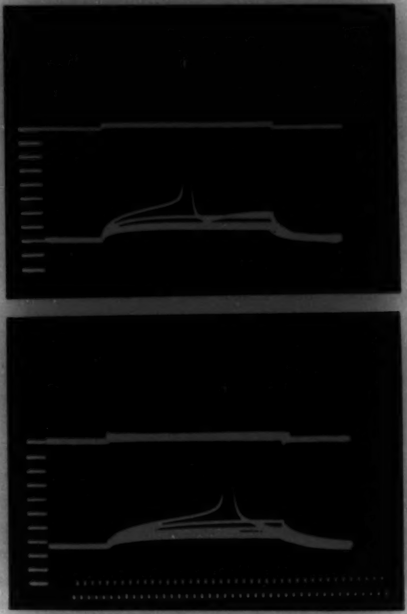

Fig. 9. A stimulating electric current (upper line in each tracing) causes membrane excitation, i.e. an action potential, in a 14-days denervated fibre (lower record). For comparison, the upper record shows a similar experiment in an innervated muscle. Time marker 1000 c/s; voltage calibration 10 mV steps.

TABLE 2. The potential difference across the membrane at which excitation occurs in fibres of the tenuissimus muscle. The S.E.'s of the means are shown

Days of denervation	No. of fibres	Critical membrane potential (mV)
0	34	$48 \pm 0{\cdot}6$
7	39	$47 \pm 0{\cdot}8$
14	62	$51 \pm 0{\cdot}6$

resistance, and it follows that to produce the same potential reduction between *A* and *B* the drop in resistance for a localized short circuit has to be greater than for a more generalized one.

In order to produce a depolarization at the innervated end-plate it is necessary to reduce the total effective resistance between the inside and the external medium. For this particular purpose this resistance can be considered as made up of two components in parallel, the resistance of the end-plate membrane and a shunt resistance due to structures outside the end-plate. The magnitude of the latter resistance is a function of the core conductor properties of the muscle fibre. Since normally only the end-plate membrane is affected by ACh it follows that a relatively larger reduction of the resistance in the end-plate membrane is needed in order to produce a given decrease of the total effective resistance.

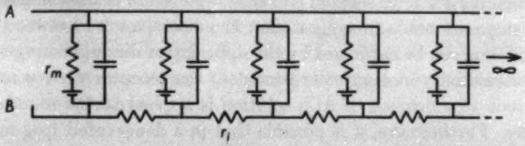

Fig. 10. A diagram of an electric model with cable conductor properties similar to those of a muscle fibre: r_m = membrane resistance; r_l = longitudinal resistance of myoplasm.

It is possible to calculate the degree of supersensitivity that can be expected from this mechanism if we know the membrane resistance, the area of end-plate membrane and the total effective resistance ($\frac{1}{2}/\sqrt{r_m \times r_1}$), where r_m = transverse resistance of the membrane times unit length; r_1 = longitudinal resistance of the fibre per unit length). For example, if the total effective resistance is 0·5 MΩ and the resistance of the end-plate membrane 100 MΩ, it is necessary to reduce the resistance of the end-plate membrane to 2 MΩ in order to decrease the total effective resistance by 20%. On the other hand, in the denervated muscle fibre the same effect will result simply when the membrane resistance decreases by 20%. Hence, in this example a supersensitivity of 40 times would result.

The ratio of the decrease in the resistances discussed is an index of supersensitivity only when the dose–response relation of ACh is linear. This is, however, presumably not the case, since it has been shown that the relation between ACh dose and membrane depolarization has an 'S-shaped' rather than linear start (Katz & Thesleff, 1957*a*). Such a non-linear dose–response relation is likely to augment further the difference in sensitivity between innervated and denervated fibres. With the membrane constants obtained for the tenuissimus muscle (Boyd & Martin, 1956*b*) it can be estimated that the sensitivity of this muscle to ACh should, after denervation, increase by a factor of 10–500, depending upon whether 100 or 20 μ is taken as the diameter of the active end-plate membrane.

The possibility that denervation supersensitivity can be explained by an increase of the receptor surface has not previously received attention. The prevailing hypothesis has been that, after denervation, the sensitivity of ACh receptors at the end-plate increased, leaving the rest of the membrane relatively unaffected. This view originated from results obtained by Kuffler (1943). He applied droplets (0·3 μl.) of ACh solution to single frog muscle fibres, and observed that after denervation the sensitivity of the end-plate region to ACh increased 1000 to 100,000 times while that of other parts showed little change. In denervated mammalian muscles we have been unable to observe a selective supersensitivity of end-plate receptors to ACh, but we have not examined chronically denervated frog muscle. Assuming, however, that the denervation phenomenon is of the same nature in mammals and in the frog it seems reasonable to suggest that the discrepancies between Kuffler's results and ours can be explained by the difficulty of distinguishing between a true supersensitivity and an enlargement of the receptor area when a large volume, such as a droplet, of ACh solution is applied to the membrane of a single fibre. Furthermore, it is possible that in a denervated frog muscle the increase in size of the receptor area is smaller than in a mammalian muscle and probably never affects more than a part of the membrane.

It is obvious that when a large part or the whole of a muscle becomes uniformly sensitive to ACh the observed degree of supersensitivity will vary according to the mode of ACh application. Close arterial injections, for instance, cannot give a true estimate of supersensitivity, nor are results obtained by local and by bath application strictly comparable.

As previously mentioned, the observed extension of ACh sensitivity can account for a several hundred times increase in the responsiveness of a denervated mammalian muscle fibre to applied ACh. This process is therefore, in our opinion, an adequate explanation for what is known as denervation supersensitivity. It is, however, likely that other factors, e.g. a lack of cholinesterase and an increased electrical excitability, also contribute to the phenomenon. Whether our results explain denervation supersensitivity in other organs remains to be shown. Considering the close similarity of this process in various types of tissues it would, however, be rather surprising if the mechanism varied markedly.

Brown (1937), and subsequently Rosenblueth & Luco (1937), showed that close arterial injection of ACh in a chronically denervated mammalian muscle produced, after an initial burst of twitches, an electrically silent contracture. In an innervated muscle ACh caused muscle twitches but never an increase in tension. Observations made in the present investigation (see also Zaimis, 1954) offer an explanation for the contracture caused by ACh in denervated muscles. The spread of the receptor area to embrace the whole membrane will allow ACh to depolarize the entire length of a muscle fibre and thereby to produce a contracture unaccompanied by conducted responses. This type of contracture is thus similar to that elicited by direct current or by a high external potassium concentration.

It is of interest that the size of the receptor area in a muscle fibre is determined by external influences. Reduced muscular activity after denervation could be such an external factor, but this is rather improbable, since it has been shown that disuse atrophy of muscle is not in itself accompanied by supersensitivity. An increased sensitivity to the transmitter agent and other chemicals is only observed when there is a lack of a living nerve connexion (Eccles, 1941). Hence, there appears to be a true influence of the nerve on the muscle membrane (cf. nervous control of muscle differentiation, Buller, Eccles & Eccles, 1958). Such an influence is probably exerted by the transmitter agent or by some other chemical substance released from nerve terminals. This is a new and particularly challenging aspect, since it points to the possibility that presynaptic activity can control the size of the chemically sensitive region in post-synaptic membranes.

SUMMARY

1. The sensitivity to acetylcholine (ACh) of chronically denervated mammalian skeletal muscle was studied by iontophoretic micro-application of the agent to single muscle fibres. The membrane potential of the fibres was simultaneously recorded with an intracellular electrode inserted close to the point of ACh application.

2. In an innervated muscle ACh produces membrane depolarization only when applied to the end-plate region of a muscle fibre. The area sensitive to ACh is small and occupies only a very limited part of the muscle membrane.

3. In a denervated muscle, however, the entire muscle membrane becomes sensitive to ACh. One to two weeks after denervation the whole surface of the muscle is about as sensitive to ACh as the end-plate region, which has maintained its original responsiveness to the drug. The muscle is uniformly sensitive to ACh and wherever applied on the membrane ACh produces a depolarization of a similar magnitude and time course as when applied to the end-plate region of an innervated muscle.

4. In a denervated muscle, as at the normal end-plate, ACh causes a non-selective increase in the membrane permeability to Na and K and possibly also to other ions.

5. ACh receptors in a denervated muscle respond to drugs in a manner qualitatively similar to that of the innervated end-plate. Anticholinesterases, on the other hand, do not potentiate the effects of ACh, suggesting that the denervated muscle membrane is practically devoid of cholinesterase.

6. Although the whole membrane in a denervated muscle is sensitive to ACh, the muscle fibres remain electrically excitable.

7. It is concluded that the increase in the size of the receptor area after denervation accounts for the supersensitivity of denervated muscles to ACh and other chemical substances. Furthermore, the extension of the ACh-sensitive region to the whole muscle will allow the drug to depolarize the entire length of a fibre and thereby to produce the ACh contracture observed in denervated mammalian muscles.

The expenses of this investigation were aided by grants from the Swedish Medical Research Council, The Muscular Dystrophy Association of America, Inc. and the Air Research and Development Command, United States Air Force, through its European Office. We are indebted to Professor B. Katz and Dr A. Lundberg for valuable suggestions and criticism. Unfailing technical assistance was provided by Miss E. Adler.

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THE DISCRIMINATION OF AFTER-IMAGES

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This paper is concerned with the illusions observed, in darkness or on looking at a moderately dim uniformly illuminated surface, after part of the visual field of one or both eyes has been exposed to bright light. Those seen in darkness are usually called 'positive after-images', and are often similar in colour to the inducing light. Those seen in moderate illumination are called 'negative after-images', and are often of approximately the complementary colour. The problem of why after-images look precisely as they do is a very complex one, for the appearance is likely to depend on many factors acting together. The sequences of chemical changes which occur in receptors after light has been absorbed by the receptive pigments certainly differ between rods and cones, and probably between different classes of cones; and adaptational changes can certainly occur in nerve cells of the retina and of the brain. All these may well play a part in determining the appearance of an after-image. We give ourselves a better chance of obtaining a complete answer to part of the problem, and a securer foundation for beginning to solve the rest of it, if at present we restrict discussion to the following simple question: 'Under what conditions do two stimuli of different physical composition give indistinguishable after-images?' When we have found two such stimuli, we shall have good grounds for supposing that the structures or processes in the retina or visual pathway which are concerned in the persistence of their after-images are affected alike by them. We shall see that there are pairs of stimuli which produce identical after-images though the immediate or 'primary' sensations are very different, and other pairs which produce distinguishable after-images though the primary sensations are indistinguishable. From these observations, inferences will be made about the probable nature of the lasting change responsible for the persistence of after-images, and about some properties of the receptive pigments of human foveal cones.

METHODS

Figure 1*a* shows the very simple apparatus used in the experiments of Part I and in the preliminary experiments of Part II (p. 198). The apparatus used in the accurate experiments of Part II (p. 199) to provide the same quantity of light (integral of luminance with respect to time) either in one short flash or in a series of flashes distributed over a longer time, is shown in Fig. 1*b*. When very brief stimuli were required, in the experiments of Part II (p. 199), two Mazda FA5 krypton-filled flash tubes were arranged as in Fig. 1*c*. Each was connected to a 50 μF condenser charged to 2200 V. The condensers were charged from the same power supply through separate resistors of 70,000 Ω; thus on suddenly discharging one, the initial rate of discharge of the other into it corresponded to a time constant of more than 7 sec. The flash tubes were triggered by separate induction coils, the primary circuits of which were broken by a Lucas spring contact breaker, giving an interval which could be continuously adjusted in the range 0–32 msec, and

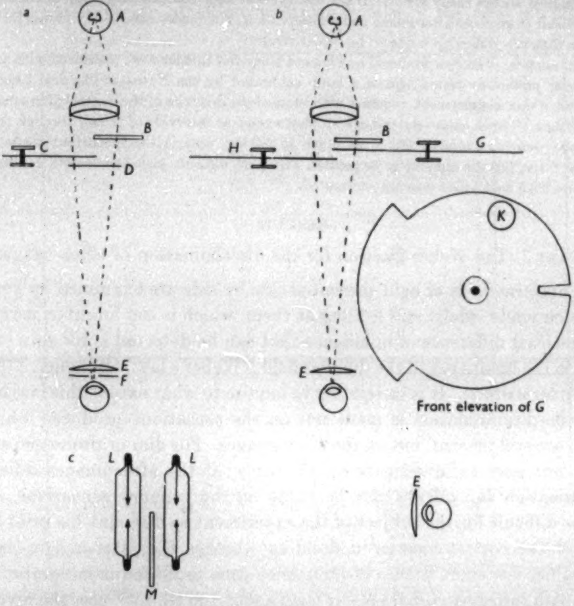

Fig. 1*a*. Plan of the apparatus used in Part I. *b*, plan of the apparatus used in Part II (p. 198), with front elevation of the double pendulum shutter. *c*, side elevation of the apparatus used in Part II (p. 199). *A*, ribbon filament lamp; *B*, neutral filter or wedge, which could be moved to cover either half of the field, or both halves; *C*, simple pendulum shutter, giving an exposure of 0·112 sec—it could be moved to cover either half of the field or both halves; *D*, camera shutter, adjustable to give various exposures; *E*, spectacle lens correcting the refraction of the homatropinized eye for the required distance; *F*, 4 mm artificial pupil; *G*, double pendulum shutter giving exposures of 15·7 msec and 1·68 sec; *H*, continuously rotating 1/107 sectored disk; *K*, weight; *L*, Mazda FA5 flash tube; *M*, brass sheet.

set with an accuracy of about ±20 μsec. Of the light in each flash, as measured with a vacuum photocell and cathode-ray oscilloscope, 95% was emitted within 200 μsec.

In nearly all the experiments the subject's pupil was dilated and fixed with homatropine. In the experiments of Part I an artificial pupil of 4 mm diameter was used. Elsewhere light was admitted through the whole of the natural pupil.

To examine the after-image the usual procedure was to turn the eyes towards a uniform white surface, and alternately to open and close that eye which had received the inducing stimulus, the other eye being continuously covered. In this way the after-image, alternately negative and positive, remained always easily visible. If the test eye was kept continuously open or closed, it was more difficult to keep the after-image under observation. The reader can readily obtain for himself evidence that this difficulty is due to binocular rivalry.

The luminances of flashes produced by filament lamp and shutter were measured with an S.E.I. photometer previously tested against a lamp calibrated by the National Physical Laboratory. The errors of this measurement, together with those of the densities of the neutral filters (measured with a Hilger Uvispek photo-electric spectrophotometer at intervals of 20 mμ through the spectrum) may amount cumulatively to as much as 0·2 log. unit in the important range around 10^6 cd. m^{-2}.sec, but are unlikely to be greater. The more difficult task of absolute calibration of the Mazda FA5 flash tubes was not attempted.

RESULTS

PART I. The Weber fraction for the discrimination of after-images

If two uniform fields of light presented side by side are examined by eye, and a decision made, whilst still looking at them, which is the brighter, it is found that the least difference of luminance that can be detected is roughly proportional to the luminance of the dimmer field ('Weber's law': Bouguer, 1760 and many later writers). It is interesting to inquire to what extent this law is valid when the discrimination is made not on the sensations produced while the stimuli are still present, but on the after-images. For dim or prolonged stimuli this is not easy to investigate satisfactorily; if the after-images differ, the discrimination can always also be made on the primary sensations, and it may be difficult for the subject of the experiment to discount his prior knowledge of the correct answer in deciding whether the after-images are discriminable. For short flashes of luminance-time product (or integral of luminance with respect to time) greater than about 100 cd. m^{-2}.sec, the investigation becomes easier; such flashes, even when they differ sufficiently to be easily discriminated on their after-images, produce indistinguishable primary sensations. The ratio of the least difference in luminance that can be detected to the luminance of the less bright field ('Weber fraction') is only slightly greater for these bright flashes, discriminated on their after-images, than for dim flashes of similar duration discriminated on the primary sensations, provided that, in the after-image experiment, the luminance-time product does not exceed about 3×10^5 cd. m^{-2}.sec; Weber's law, as can be seen in Fig. 2, is approximately valid over the very large range from

$$3 \times 10^{-3} \text{ to } 3 \times 10^5 \text{ cd. m}^{-2}\text{.sec.}$$

The increase in the Weber fraction seen in Fig. 2 at very low luminances has long been familiar; even in Bouguer's book published posthumously in 1760 there is indirect reference to it. The more conspicuous increase for very bright flashes has not previously been reported, though a closely related phenomenon was discovered, whilst investigating a military technical problem, by J. L. Russell of the Royal Aircraft Establishment, Farnborough, Hampshire, in 1937. The amount of light required to produce it is sufficient to cause a photochemical change in a large fraction of the molecules of visual pigment in the region of retina on which it falls, if the relevant pigments have photosensitivities similar to those measured objectively in the human fovea (Rushton, 1958) or to that inferred for the red-receptive pigment from the disturbance of colour matches by adaptation (Brindley, 1955). Making due allowance for uncertainties in the absolute photometric calibrations and in the corrections for size of pupil and spectral distribution of the light, we can say roughly that if the pigments have the properties described by Rushton (1958), bleaching of 85% of the molecules can, and of 99·0% cannot, be distinguished from bleaching of 99·99% of them; if they have the properties

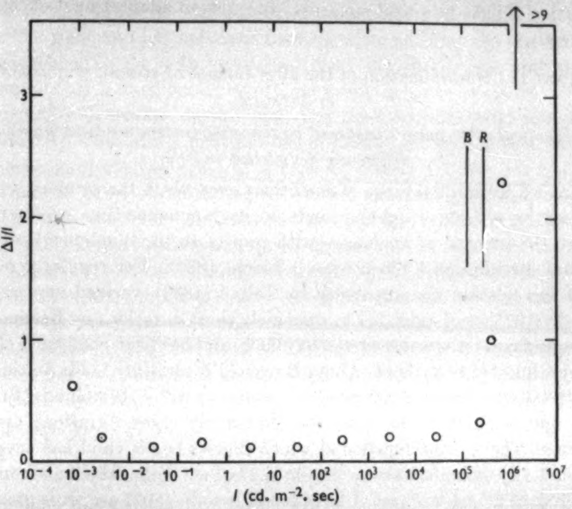

Fig. 2. The Weber fraction $\Delta I/I$ for brief flashes of light. Artificial pupil 4 mm; field diameter 2°; duration of stimulus 0·112 sec, except for the strongest stimulus (not discriminated from one 10 times as bright); this was of 0·63 sec. The discriminations were made on the primary sensations up to and including 31 cd. m^{-2}.sec, and on the after-images above this. The vertical line *B* shows the amount of light required to bleach all but 1/e of the red-receptive pigment of the fovea, estimated from the subjective measurements of Brindley (1955), and the vertical line *R* a similar estimate from the objective measurements of Rushton (1958).

inferred by Brindley (1955), bleaching of 99·0% can, and of 99·99% cannot, be distinguished from bleaching of all but one in 10^8. These fractions are so large as to make it very probable that the cause of the observed increase in the Weber fraction is the bleaching of most of the visual pigment, and not the saturation of some later stage in the transmission of visual information. This conclusion is further strengthened by the evidence, to be given in Part II, that the persistence of after-images under conditions closely similar to those of the experiments of this Part depends on chemical events which follow the absorption of light by visual pigments, and not on adaptational effects upon nerve cells.

PART II. Discrimination of the after-images of stimuli which differ in duration

Identical after-images produced by the same luminance-time product differently distributed in time

There is a substantial range of conditions over which the primary sensation produced by a flash of light depends on its luminance-time product (or in general, the integral of luminance with respect to time) independently of its temporal distribution ('Bloch's law': Bloch, 1885). For regularly repeated flashes this relation was discovered by Talbot (1834), verified very critically by Hyde (1906), and extended to extremely short durations by Beams (1934). For single flashes it was discovered by Bloch, and has been confirmed at threshold by a number of authors. Above threshold it certainly holds for durations down to $4{\cdot}1 \times 10^{-7}$ sec and retinal illuminations up to 3×10^{8} trolands (Brindley, 1952), and is likely to be valid for indefinitely short durations and high intensities. The *greatest* duration at which Bloch's law is valid has never been systematically examined above threshold. For a 3° foveal field at luminance-time product 0·5 cd. m^{-2}.sec, I have found it to be 0·027 sec; it is greater for dimmer flashes, and may perhaps be smaller for brighter ones.

For after-images, preliminary experiments to investigate the conditions under which Bloch's law holds, using the crude but flexible apparatus of Fig. 1*a*, indicated that for stimuli lasting not more than about 2 sec the whole course of the positive or negative after-image, excluding its first 15 sec, was determined by the total quantity of light in the stimulus, independently of its distribution in time. For stimuli lasting between 2 and 5 sec, the same was very nearly but not quite exactly true. The apparatus shown in Fig. 1*b* was built to confirm this finding accurately over a limited range of conditions. The left half of the circular photometric field subtending 3° at the eye was illuminated with a single flash of duration 15·7 msec. The shutter which determined this flash also exposed the right half of the field for 1·68 sec, the 15·7 msec flash occurring in the middle of this exposure; but light reached the subject's eye from the right half of the field only during a fraction 1/107 of the 1·68 sec during which it was exposed, i.e. a total of 15·7 msec, because of the rotating sectored disk interposed between the source and this part of the shutter. Thus if no filters were inserted, or the same filters in each path, the integral of luminance with respect to time was the same for the right half of the field as for the left at all wave-lengths, but the temporal distribution was very different.

Experiments with this apparatus very clearly confirmed the preliminary ones. At 8×10^4 cd. m^{-2}.sec, with the sectored disk rotating at any speed between 15 and 40 rev/sec, the after-images, excluding their first 15 sec, were alike in the right and left halves of the field when no filter was inserted. If a filter was placed in the path for one half-field, the after-image, both positive (i.e. seen against a dark background) and negative (i.e. seen against a bright background) was weaker on this side. Practised subjects could consistently discriminate in this way a neutral filter of density 0·1 on one side. Even to unpractised subjects the effect of a filter of density 0·2 was very obvious. The sensations experienced during the stimuli and during the first second or two after them were very different for the right and left halves of the field, whether the sectored disk was rotated at more or at less than the flicker fusion frequency; and the relative amounts of light delivered on the two sides could not, even after much practice, be estimated from these primary sensations with the accuracy that was very easily achieved by waiting for 15 sec and then examining the after-images. During the first 15 sec the after-images of the two halves of the field usually differed in colour, and no adjustment of the relative luminances by adding neutral filters would cause them to match perfectly.

Deviations from Bloch's law at very short durations

Since Bloch's law holds for after-images in the range from 15 msec to 2 sec or so, it might be expected, by analogy with the corresponding law for the primary sensations, that it would hold also at very much shorter times. For stimuli of less than 10^5 cd. m^{-2}.sec, the available evidence does not contradict this expectation. For extremely bright stimuli, however, it is certainly false. Two Mazda FA 5 flash tubes were set up as shown in Fig. 1*c*, and discharged at an interval of 0·28 msec. The upper and lower halves of the after-image produced were found to be distinguishable; the 240-J flash from the nearer tube was evidently bright enough to produce a saturation similar to that found in the experiment of Fig. 2, and the additional light from the further tube made no difference to the after-image.

The interval between the flashes was increased to 4 msec. The upper and lower halves of the after-image were then found to be clearly different; whether seen against a dark background or a bright one, the upper half, where light had been received from both tubes, was stronger than the lower half, where light had been received only from the nearer tube. Intervals intermediate between 0·28 and 4 msec gave intermediate results, the upper and lower halves of the after-image being neither as easily distinguished as at 4 msec nor as clearly alike as at 0·28 msec.

All the five subjects tested with this apparatus could consistently distinguish, without sensory clues other than the after-image, whether the tubes had been fired 4 msec or 0·28 msec apart. For three of them the above description applies exactly. For the other two subjects the upper and lower halves of the after-image were not quite indistinguishable at 0·28 msec, but the difference then seen was very much smaller than at 4 msec. Provided that certain controls are satisfied, these findings imply a deviation from Bloch's law: two flashes separated by 4 msec give an after-image which differs from that seen when the same two flashes are separated by only 0·28 msec.

Controls. When the time course of the flashes was recorded with a vacuum photocell and cathode-ray oscilloscope, no influence of either flash on the intensity or duration of the other could be detected. In each flash 95% of the light was emitted within 0·20 msec. Thus when the farther tube was fired first, as was usually done (though the same results were obtained when it was fired second) almost all the light from it had been emitted before the nearer tube, through which the light had to pass to reach the subject's eye, had been activated. The question of whether the transparency of the nearer tube alters after its activation need not, therefore, be considered.

DISCUSSION

There is good theoretical reason to expect that, for durations short compared with that required for substantial regeneration of the photosensitive substance, the extent of a photochemical reaction should be determined by the total amount of light received, independently of its distribution in time. At very short durations this relation certainly breaks down for rhodopsin and probably for human foveal pigments (see below); but between these very short durations and the 5 sec or so that are probably required for any substantial degree of regeneration, it would be more surprising to find it false than true for a human receptive pigment, especially since it has been verified in part of the relevant range by Campbell & Rushton (1955) for rhodopsin and by Rushton (1958) for foveal pigments. The finding that a given amount of light produces the same after-image (except for the first 15 sec), whether it is delivered within 15·7 msec or spread over 1·68 sec, is thus fully consistent with the hypothesis that the after-image of a brief stimulus, from the fifteenth second until its disappearance 100–300 sec later, depends upon photochemical effects, and not at all upon adaptation or potentiation of neural mechanisms in the retina or brain as a result of the intense activity that presumably occurs during and immediately after the stimulus. The fairly close agreement between the time of persistence of after-images and the time necessary for the regeneration of foveal pigments (Brindley, 1955; Rushton, 1958) suggests further that the relevant photochemical effect, i.e. the condition whose presence determines that an after-image shall be observable, is either the absence of molecules of receptive pigment from the places where they should be, or the presence of primary or secondary products of photolysis which during regeneration disappear by being reconverted to receptive pigment.

To what extent are alternative hypotheses admissible? The sensations produced by the 15·7 msec and the 1·68 sec stimuli are very different indeed. So also, if we may extrapolate slightly from Ronchi & Moreland (1957), are their electroretinograms, which probably represent the electrical activity of the rods and cones (see Brindley, 1959). None of the very many kinds of retinal ganglion cells whose electrical activity has been recorded would be likely to give similar responses to stimuli differing as these do, and the same applies to the less abundant information available about spike discharges from the inner nuclear layer (Brindley, 1956; Brown & Wiesel, 1958) and about intra-retinal slow electrical activity (Tomita, 1950; and later writers). If, then, adaptation or potentiation of nerve cells is concerned, it must either be confined to cells of a kind not yet detected electrically in experimental animals, or depend on a functional process in them that is scarcely at all correlated with their electrical activity. These ways of escape are not easy to accept, and the evidence seems strong that the persistence of the after-images of brief stimuli, excluding the first 15 sec, depends solely on the chemical consequences, within the receptors, of the absorption of light by the receptive pigments.

The inequality of the after-images during the first 15 sec is most reasonably explained by supposing that neural effects are then contributing. In the first 5 sec or so the inequality is very great, as if neural effects then much predominate over photochemical ones. After the fifth second it diminishes swiftly, so that at 10 sec the after-images are nearly alike, and at 15 exactly so.

It would be rash to extrapolate far beyond the conditions of the present experiments. Strong though I believe the evidence to be for the purely receptoral origin of the after-images of *brief* stimuli, it does not follow that neural factors are not concerned when the stimuli are longer. Nevertheless, the possibility, even under a limited range of conditions, of producing after-images whose place of origin is known and fairly simple, provides a new tool for investigating the function of the human retina.

The deviation from Bloch's law found at very short durations

This is almost certainly related to a phenomenon discovered by Hagins (1955). Hagins measured by reflexion densitometry the amount of rhodopsin in the eyes of albino rabbits before and after exposing them to a flash lasting less than 1 msec. He found that a single flash, however bright, never bleached more than half the rhodopsin present; but a second flash, if delivered several tens of milliseconds later, would bleach half the remainder. Two plausible explanations can be suggested: absorption of a quantum of light may bring a molecule of rhodopsin into a state from which, during the following few milliseconds, it can, with about equal probability, be bleached or return to its original state; or light may not only convert rhodopsin into a substance X, but also convert X into rhodopsin. In the second explanation, it must be assumed that X spontaneously loses, within a few milliseconds, the capacity for being reconverted into rhodopsin by light. The work of Hubbard & Kropf (1958) makes it probable that the second explanation is correct, and that X is a compound of opsin with all-*trans* retinene, or perhaps a mixture of compounds of it with retinene isomers other than 11-*cis* and 9-*cis*.

The deviation from Bloch's law shown by human foveal after-images when the inducing stimuli are very short can be completely explained by supposing that the receptive pigments of the foveal cones share this property. If so, the time required for the dark reaction which restores part of the manifest photosensitivity after a flash must be between about 0·4 and 4 msec.

SUMMARY

1. For the discrimination of the after-images of brief flashes, Weber's law, $\Delta I/I =$ constant, holds approximately up to 3×10^5 cd. m^{-2}.sec. At higher intensities the Weber fraction $\Delta I/I$ increases very much. This 'saturation' of discrimination is probably due to bleaching of nearly all the photosensitive pigment.

2. The after-images (excluding their first 15 sec) produced by stimuli of duration between 15·7 msec and 1·68 sec (and approximately up to 5 sec) are determined by the total quantity of light delivered, independently of its distribution in time. This is the counterpart, for after-images, of Bloch's law. From this it is argued that the persistence of after-images under these conditions depends on the chemical consequences, within the receptors, of the absorption of light, and not on adaptational changes in nerve cells.

3. For very short flashes (0·4–4 msec), a conspicuous deviation from Bloch's law occurs at high intensities but not at low. A closely analogous property of rhodopsin was discovered by Hagins (1955), and it is suggested that the receptive pigments of human foveal cones resemble rhodopsin in this respect.

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THE COLOUR OF MONOCHROMATIC LIGHT WHEN PASSED INTO THE HUMAN RETINA FROM BEHIND

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Light can cause a visual sensation only if it is absorbed by one or more photosensitive pigments in the retina. The only photosensitive pigment which has been extracted from the eye of any mammal is rhodopsin, or visual purple. This substance can be seen to be present in the rods, and its absorption spectrum corresponds closely to the spectral sensitivity of the eye in dim light, where rods are believed to be the only receptors active. In dim light ('scotopic vision'), colours cannot be discriminated, which accords with the hypothesis that only one class of receptors is concerned. In bright light ('photopic vision'), on the other hand, colours can be discriminated and the colour-matching properties of the normal human eye demand that at least three classes of receptors be present. It is commonly assumed that these three classes of receptors contain different photosensitive pigments, whose absorption spectra correspond closely to the spectral sensitivities of the receptors in which they are contained, as the absorption spectrum of rhodopsin does to the spectral sensitivity of rods. However, an alternative hypothesis has been several times suggested, which makes the basis of human colour vision similar to that which is very probably present in birds.

In birds the inner segments of the cones contain coloured oil droplets through which the light must pass to reach the outer segment where the visual pigment is probably situated (Schultze, 1866, and earlier writers). The droplets are deep red, orange, yellow and colourless, and evidently might, by acting as colour filters, provide a basis for colour vision even if all the cones contained the same receptive pigment. This is probably the situation in the domestic fowl, from whose retina Wald (1937) and Wald, Brown & Smith (1955) extracted two photosensitive pigments, rhodopsin and a new pigment, iodopsin, which is likely to be the single receptive pigment of the cones.

No coloured filters have been seen in the cones of mammals, but this is not sure proof that they do not exist in the living eye; and there are other hypotheses which would allow human colour discrimination to depend on a single receptive pigment. There might be in each cone an interference filter, or else a structure analogous to a wave guide, which selects (in part) the light admitted to the place where the pigment is situated. We need not consider the details of these suggestions, for there is one simple test by which, in our opinion, they are all shown to be very unlikely.

If the distinction between one cone and another is due to colour-selective mechanisms situated in front of the visual pigment, then there will be no distinction between them when light falls upon the retina from behind; hence in this case colours cannot be discriminated. If, on the other hand, different cones contain different receptive pigments without any accessory mechanism selecting the light which reaches them, then any monochromatic light will stimulate the various cones in proportions which depend on the absorption spectra of their pigments, but not on the direction from which the light comes; hence such a light will appear to be of the same colour, whether it is sent in the normal manner through the cornea, or through the sclera so that it falls on the receptors from behind. We have tested these inferences, and found the first to be very clearly false, and the second to be near the truth.

EXPERIMENT

If the reader closes his eye, presses upon the eyeball at the temporal margin of the orbit, and turns this eye to look at the bridge of the nose, the pressure phosphene is readily seen as a round patch which moves with the finger. If the small light bulb of an ophthalmoscope is used as pressing object, then, when the light is switched on, the locality of the phosphene is occupied by a diffuse red patch of light. It would be expected to be red because, as Schouten (1934) has shown, the other wave-lengths are largely absorbed by blood and melanin as the light transverses the choroid. It is therefore necessary to use monochromatic lights if colour judgements are to be significant.

Qualitative observations throughout the long-wave half of the spectrum were made as follows. Monochromatic light was obtained from a large Stiles' double monochromator. Light was 'piped' from the exit slit to the eye through a short glass rod whose end was ground obliquely to lie flat against the sclera. It was pushed as far as practicable into the temporal fornix of the conjunctival sac, which was anaesthetized with cocaine. The eye was rotated as far as possible in a nasal direction; the light then fell about 30° from the fovea.

For all wave-lengths greater than 520 mμ it was obvious that monochromatic light had almost the same apparent colour, whether it reached the retina from the sclera or from the pupil. Lights seen trans-sclerally appeared somewhat less saturated, and at wave-lengths shorter than 500 mμ they could only be seen by rod vision. This is what might be expected in view of the great absorption of these wave-lengths in the choroid.

Quantitative measurements at two wave-lengths were made using the

579 + 577 mμ and 546 mμ lines of the Hg spectrum, isolated from the other lines by suitable filters and applied to the eye by the glass 'light pipe' as before. It is important that the comparison field from in front should fall upon a neighbouring region of retina since it is known that the appearance of a coloured light depends upon its retinal location. Upon the side of the nose was stuck either a piece of white adhesive tape upon which a spectral patch was projected or a mirror in which a spectral patch was observed; by this device the trans-scleral and comparison areas were caused to lie side by side in the visual field.

TABLE 1. Wave-length (mμ) of monochromatic light from in front which matches Hg line incident from behind

Hg line	Too red (or yellow)	Too green (or blue)	Mean match
579 + 577	581	577	579
546	554	548	551

The subject was presented with a monochromatic comparison field whose wave-length could be varied, and he said whether it was too red (or yellow) or too green (or blue). The result of a typical experiment is given in Table 1. There was always a small discrepancy, as shown, less for yellow light than for green. This difference in colour of a monochromatic light when falling upon the retina from front or back is the same in sense and similar in magnitude to the colour change which Stiles (1937) observed upon the fovea when the point of entry of the beam was shifted from the centre to the periphery of the pupil, though he found that the discrepancy was greater for yellow light than for green. The colour difference which appears when light falls on the retina from behind is so small that it is consistent with the view that the colour mechanism of the cones derives from several distinct pigments, and is not easy to explain by colour selectors in front of a single pigment.

The relative rod: cone threshold

The foregoing observations suggested that the relative rod: cone excitability might be greater for trans-scleral illumination than for illumination through the pupil. This also was to be expected from the Stiles-Crawford effect and from the structure of rods and peripheral cones, since cones probably funnel light on to the visual pigment much better in illumination from the front than from the back, and with rods this phenomenon should be less marked. Determinations were made as follows.

The subject's head was fixed by biting upon a dental impression and light of wave-length 546 mμ from a Hg lamp was arranged to pass trans-sclerally by way of the glass rod which was clamped in place. Light of 551 mμ (which appeared the same colour) was projected upon a white patch on the nose and the two lights were seen upon adjacent retinal regions. The lights were exposed alternately since in the periphery it is hard to judge the intensity of a steadily maintained illumination. The subject instructed the operator to change the intensity of the light from the monochromator until the two appeared equal. A density of 2 log. units was then interposed in each beam; this brought both lights well into the scotopic range, and a new brightness match was made. The first match was then repeated.

The work requires considerable control both of head and of fully deflected eyes in order to keep a steady contact with the glass rod, and we found the experiment difficult to do accurately. The result obtained, however, was

$$\frac{\text{rod threshold (front)}}{\text{rod threshold (back)}} \div \frac{\text{cone threshold (front)}}{\text{cone threshold (back)}} \doteqdot 4$$

The value probably lies between 2 and 8.

This is less than we had expected either from the Stiles–Crawford differences between rods and cones or from Polyak's (1941) description of the relative embedding of receptors in the pigment epithelium.

The brightness of the retina in trans-scleral illumination

When the retina is illuminated from behind as described, part of the light which has traversed the retina passes through the pupil and falls upon the bridge of the nose. If the pupil is fully dilated with homatropine and cocaine and a small mirror is interposed between nose and eye the rays may be reflected back into the eye and fall either upon the fovea or near to the bright patch itself, according to the tilt of the mirror.

By placing the reflected patch on the fovea, the subject can examine part of his own trans-sclerally lit fundus, and can see retinal and choroidal blood vessels. By placing the reflected patch close to the trans-scleral patch, he can assess their relative brightness; the trans-scleral patch appears substantially brighter. A quantitative estimate of the relative brightness was made by the following method, in which an external observer instead of the subject himself viewed the reflected patch.

The subject first made a match in colour and brightness between the trans-scleral patch and an illuminated card viewed in the nasal mirror, the inclination of the mirror being such as to bring the two fields to be compared upon neighbouring areas of the retina. He next tilted the mirror so that he saw the eye of the observer coinciding with the trans-scleral patch. The observer could then see the trans-sclerally illuminated part of the subject's fundus in the mirror and could find by what factor the illumination of the card had to be diminished in order to match it. The factor was found to be about four.

Since the reflected light has suffered transmission losses amounting to an attenuation of perhaps 2·5 times in the double traverse of the vitreous, lens and cornea, we estimate that a patch of retina illuminated from behind appears roughly 1·6 times as bright subjectively as an equal area of retina which receives from in front as much light as reaches the fully dilated pupil from the trans-sclerally illuminated patch.

CONCLUSIONS

These results are consistent with the view that colour vision depends upon the presence in the cones of several visual pigments with only a second-order effect due to wave guides, etc. (the Stiles retinal directional effect). The results are not consistent with a single visual pigment in the cones with accessory colour selecting devices, unless one of the following two (improbable) assumptions is made. (*a*) The colour selectors are of such a nature and situation that they operate in almost exactly the same way to light falling upon the retina from behind as from in front. (*b*) Cones are inexcitable to light from the back and in our experiments (contrary to our belief) they were always excited by light entering from the front by the usual paths and hence through the normal colour selectors. This could only happen if in front of the cones there were reflectors sending back more light than did a mirror at the pupil.

SUMMARY

1. Human colour vision must depend upon at least three different photoreceptor systems.

2. There might be either (*a*) three or more different photosensitive pigments; or (*b*) only one such pigment, but various colour selectors interposed between it and the light.

3. Monochromatic light reaching the retina from the back would miss the colour selectors; thus lights of all wave-lengths would appear the same hue if 2(*b*) were true, but they would look their usual colour if 2(*a*) were true.

4. They look their usual colour.

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A STUDY WITH THE ELECTROMAGNETIC FLOWMETER OF FLOW PATTERNS IN CAROTID AND FEMORAL ARTERIES OF RABBITS AND DOGS

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The relationship between blood pressure and the rate of flow of the blood during the cardiac cycle is one of the most fundamental problems in haemodynamics. The electromagnetic flowmeter is an instrument that not only offers a convenient means of studying the phasic blood flow, but also seems, on theoretical grounds, to be almost ideal. Reliable reports concerning the investigations on phasic changes of rate of blood flow in the cardiac cycle (i.e. the flow pulse) are scarce, especially systemic records by the electromagnetic method (Katz & Kolin, 1938; Richards & Williams, 1953). On the other hand, as has already been discussed by Shipley, Gregg & Schroeder (1943) and by McDonald (1955), there are some discrepancies between patterns of flow pulse recorded by the electromagnetic flowmeter and those recorded by other methods. We have therefore re-examined the reliability of the electromagnetic method in recording phasic flow changes both *in vitro* and in experimental animals. The results of the experiments *in vitro* have already been published (Inouye & Kuga, 1954) but records in animals were presented only in a preliminary form in that report. The present paper is concerned principally with the pattern of pulsatile flow recorded by our electromagnetic flowmeter in rabbits and dogs under nearly normal circulatory conditions.

METHODS

Rabbits weighing 2–3 kg were used to observe carotid flow. A carotid artery was exposed under local anaesthesia, the whole area having been previously infiltrated with cocaine hydrochloride. The femoral arteries were either too narrow to apply our cannulating method or the blood flow through them was too small to allow accurate measurement. Observations of femoral flow were, therefore, made on dogs weighing 5–14 kg, which were anaesthetized by intramuscular injection of 'Ouropan-soda' (*N*-methyl-cyclohexenyl-methyl barbiturate) in a dose of 0·1–0·2 g/kg. An additional injection of a dose of 0·1–0·2 g/kg was made during experiments when necessary. Clotting of the blood was prevented by heparin.

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Experimental procedures to record pressure and patterns of flow pulse were the same as previously described (Inouye & Kuga, 1954), i.e. a membrane manometer and an electromagnetic flowmeter of a.c. type inserted into the blood vessel after heparinization. But in a few later cases a transducer manometer, or a flowmeter of d.c. type (Inouye, Kuga & Usui, 1955) was employed. The flow patterns were recorded from the flowmeter on a cathode-ray oscilloscope with a simple moving-film type of camera. The records from the two types of flowmeter were almost identical (Kosaka, 1956).

In this paper we shall regard the flow patterns thus obtained from the experiments on animals without any alteration of their circulatory conditions, other than those due to the procedures necessary for recording them, as 'normal' patterns.

RESULTS

The zero level of flow was first recorded by clamping the artery for a few seconds. When the clamp was released, the initial two or three flow pulses were usually higher (occasionally lower, as is seen in Fig. 1) than the later ones. If the duration of occlusion was prolonged to 1–3 min, such an increased flow level continued for 30–60 sec. It had its crest at 10–15 sec after the occlusion was released, and then returned slowly to a constant level (Fig. 1). Such a phenomenon was observed both in carotid and femoral arteries. When a contralateral artery had been occluded previously this increase in the rate of flow seemed to be enhanced. These results appear to coincide with those reported by Randall & Horvath (1953), who considered them as due to reactive hyperaemia. In order to eliminate this effect we have always continued recording until the effect of reactive hyperaemia was over. Then the vessel was occluded again as a check of zero drift. Our 'normal' flow patterns have always been obtained in this way after the effects of the initial occlusion have passed. Examples of such records are shown in Fig. 2.

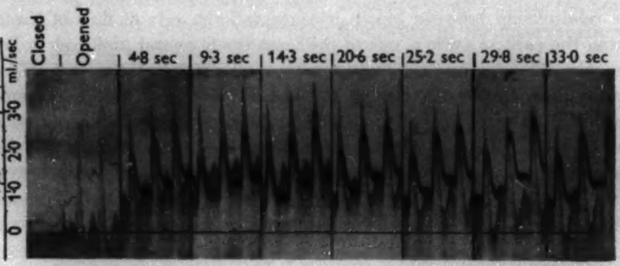

Fig. 1. Typical recording of femoral blood flow and pressure in an anaesthetized dog after 2 min occlusion of the artery. Ordinates, pressure values (mm Hg) and flow rates (ml./sec). Numbers on each record segment show time elapsed after release from occlusion of the artery.

As is shown in these figures, pulsatile blood flow increased abruptly in systole. The peak value is designated P_1. Flow then decreased rapidly and formed a relatively sharp, incisura-like trough (V_1), which was followed by a second wave in diastole. The crest and lowest point of this wave are called P_2 and V_2 respectively, in the following description. Although one or two oscillations were occasionally observed on this diastolic wave, yet the outline of the flow pulse contours might generally be described as being composed of these four components, P_1, V_1, P_2 and V_2 (cf. flow patterns of Shipley *et al.* 1943; and McDonald, 1955). These peaks and dips in the contour of the flow pulse seem at first glance to coincide with those of the pressure pulse, but the flow actually precedes the pressure, since our flowmeter has a time lag of about 10 msec in its response.

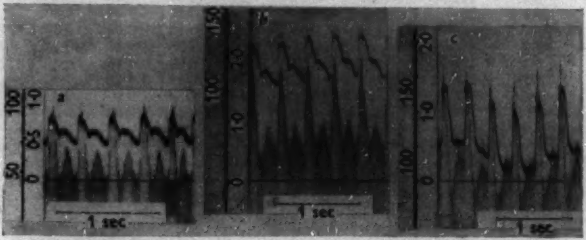

Fig. 2. Typical recordings of normal carotid and femoral blood flow and pressure. *a*, carotid flow of a rabbit (No. 10); *b*, carotid flow of a dog (No. 13); *c*, femoral flow of a dog (No. 32*a*). Ordinates: left, B.P. (mm Hg); right, blood flow rate (ml./sec).

As is seen in Figs. 1 and 2, the mean flow levels of both carotid and femoral arteries showed slow fluctuations parallel to similar changes in the pressure level (due to respiratory or other causes). Thus at the lower flow level a small retrograde flow might be seen which disappeared when the mean flow level was raised. In order to confirm the fact, pointed out by Shipley *et al.* (1943) and more recently by McDonald (1955), that the systolic back-flow component is constantly present in the femoral flow, it is necessary to take an average of their wave-like variations for a considerable length of time. We may also describe a mean flow pulse contour by obtaining mean values of the four main components P_1, V_1, P_2 and V_2 over a considerable series of pulses. Tables 1–3 were constructed for this purpose, the experimental animals being arranged in the order of their body weights. These tabulations demonstrate the following facts.

Generally speaking, the heights of P and V from the zero reference level seem to be arranged in the following order:

Carotid flow, $P_1 \quad P_2 \quad V_1 \quad V_2$,
Femoral flow, $P_1 \quad P_2 \quad V_2 \quad V_1$.

The carotid flow pulse is very similar to the form of the pressure pulse, but in the femoral pattern the order of V_1 and V_2 are reversed, with V_1 forming an incisura of considerable depth. At this point a systolic back flow frequently occurs, while in the carotid flow V_1 has at most the same order of magnitude as that of V_2 and a consistent appearance of negative systolic flow is rare.

Table 1. Flow in carotid arteries of rabbits under local anaesthesia

Animal no.	Body weight (kg)	Blood pressure (mm Hg)	Heart rate (beats/min)	Blood flow rate (ml./sec)					
				P_1	V_1	P_2	V_2	Mean	
1	2·1	97/77	221	0·51	0·16	0·23	0·19	0·27	0·29 (2·1 kg)
2	2·1	114/76	212	0·76	0·26	0·35	0·13	0·32	
3*	2·1	—	—	0·55	0·20	0·30	0·17	(0·30)	
4	2·2	111/80	226	0·47	0·21	0·27	0·18	0·28	
5	2·3	—	240	0·84	0·14	0·28	0·09	0·30	0·33 (2·5 kg)
6	2·4	99/68	189	0·70	0·02†	0·25	0·03	0·30	
7*	2·5	—	—	0·71	0·16	0·38	0·17	(0·38)	
8	2·5	121/85	243	0·71	0·23	0·37	0·13	0·35	
9	2·9	113/82	237	0·98	0·38	0·51	0·33	0·57	0·51 (3·0 kg)
10	3·0	95/76	197	0·94	0†	0·39	0·07	0·41	
11	3·1	100/71	240	1·05	0·32	0·51	0·29	0·54	

* Slow recording, hence heart rate and mean flow rate are not computed accurately. † Distinct negative flow appears in some cardiac cycles of one respiratory fluctuation period.

Table 2. Flow in carotid arteries of anaesthetized dogs

Animal no.	Body weight (kg)	Blood pressure (mm Hg)	Heart rate (beats/min)	Blood flow rate (ml./sec)					
				P_1	V_1	P_2	V_2	Mean	
12	5·4	164/126	182	1·07	0·53	*	0·41	0·65	
13	7·3	131/104	180	2·26	0·50	0·83	0·42	0·97	1·22 (7·7 kg)
14	7·7	154/123	171	2·24	*	*	1·06	1·40	
15	8·2	127/104	178	2·02	1·20	*	0·83	1·30	
16	8·7	158/119	193	1·86	1·18	1·30	0·99	1·39	1.50 (9·5 kg)
17	8·9	168/124	180	2·33	1·63	1·69	1·37	1·73	
18	9·1	151/118	184	3·99	1·10	1·84	1·15	2·04	
19	9·5	159/123	202	2·02	1·00	1·15	0·80	1·17	
20	10·0	150/108	208	2·97	0·09†	1·22	0·77	1·22	
21	10·5	174/136	200	2·51	1·04	1·38	1·00	1·46	
22	11·5	—	206	2·56	1·15	1·50	1·08	1·60	1·78 (12.3 kg)
23	12·0	—	163	1·63	0·65	0·98	0·59	0·95§	
24	12·5	145/103	192	3·80	1·62	1·96	1·12	2·02	
25	13·0	166/128	198	2·94	1·01	1·60	1·16	1·71	

* P, or P and V, are not distinct, passing gradually to the presystolic level V. † Negative flow appears in some cardiac cycles. § Omitted from the mean given in the last column.

This relation of the heights of P and V has been independent of the presence of a negative flow.

Mean flow level and amplitude of flow pulse waves seem to be related to the body weight of the animal, especially for carotid flow of unanaesthetized rabbits (see the last column of Table 1). The fact that the data on anaesthetized dogs show some inconsistency, though the effect of body size on the magnitude of flow rate is observed, might be attributable to the circumstance that it is almost impossible to anaesthetize all dogs examined to an equal extent (e.g. see No. 29, in which there is a marked bradycardia and the flow is very low). On the other hand, the mean flow levels are also in close correlation with the values of P_2, but they do not seem to be correlated with blood pressure levels.

TABLE 3. Flow in femoral arteries of anaesthetized dogs

Animal no.	Body weight (kg)	Blood pressure (mm Hg)	Heart rate (beats/min)	Blood flow rate (ml./sec)					
				P_1	V_1	P_2	V_2	Mean	
26	5·4	168/117	200	1·55	0·55	0·80	0·60	0·90	
				(1·00	0†	0·25	0·25	0·35)*	0·58 (6·0 kg)
27	5·6	163/113	166	0·84	0·22	0·38	0·26	0·40	
28	6·0	—	222	1·36	−0·16	0·34	0	0·33	
29	6·0§	162/96	97	1·42	−0·15	0·14	0·02	0·15	
30	6·9	153/80	183	1·39	0·45	0·48	0·45	0·69	
31 a	7·9	133/96	173	1·90	−0·20	0·72	0·38	0·68	(0·70 (8·4 kg)
b		134/97	180	2·54	0·22	1·00	0·62	1·05	
				(2·14	−0·18	0·60	0·22	0·65)*	
32 a	8·0	132/117	196	1·81	−0·25	0·60	0·30	0·53	
b		149/105	187	2·13	0·06†	0·77	0·63	0·84	
				(1·83	−0·24	0·47	0·33	0·54)*	
33	8·2	—	186	1·88	−0·21	0·60	0·37	0·65	
34	8·5	164/103	214	2·20	0·77	1·06	0·98	1·25	
				(1·60	0·17†	0·46	0·38	0·65)*	
35	8·7	153/116	195	1·22	−0·13	0·45	0·20	0·40	
36	9·5	145/70	161	2·80	−0·27	0·83	0·10	0·72	
37	10·0	168/116	174	1·31	0·30	0·59	0·44	0·66	0·88 (11·9 kg)
38	11·0	135/80	210	3·05	−0·45	0·67	0·40	0·80	
39	12·0	—	175	3·26	−0·32	1·08	0·78	1·14	
40	12·5	155/90	180	3·50	−0·60	1·00	0·80	1·10	
41	14·0	—	173	3·06	−0·86	0·95	0·30	0·76	

* Corrected values by raising zero reference level (see text). † Negative flow appears in some cardiac cycles. § The animal seemed to be anaesthetized excessively and the results are omitted from the mean given in the last column, together with those from No. 34.

In some cases, the femoral flow (peak and mean levels) is higher than the value predicted from that of other animals of nearly the same body size (Nos. 26 and 34) or that obtained on the same animal at other times (Nos. 31 and 32) and the negative flow component is absent, or if present is indistinct. In these cases the inconsistencies could be removed if the zero level was raised by 0·3–0·5 ml./sec. In other cases, a small lowering of the zero level seemed to be necessary as for example in the carotid flow of dogs (Nos. 17, 18 and 23), but not in that of rabbits.

Reactive hyperaemia

We did not make a systematic study of the effect of reactive hyperaemia due to occlusion of an artery, but only examined the carotid and femoral flows in a few cases. They correspond to categories 1 and 3 of Randall & Horvath (1953), and our observations appear to agree with theirs. The change in flow level due to reactive hyperaemia seemed to be related linearly to the logarithm of the time elapsed after releasing occlusion (Fig. 3). It would be expected that the general form of flow pulsation is affected by reactive hyperaemia, since a considerable alteration of the flow occurs under this condition; the back flow disappears and the diastolic flow is increased (D. A. McDonald, personal communication). In the brief rising phase of hyperaemia, when mean flow is rising rapidly, it was seen that increase in the height of P_1 was less than that of the succeeding V_1 and P_2, as is shown in Fig. 3, and hence the early diastolic dip became shallower. However, at its peak or in its slowly falling phase the heights of P_1, P_2 and V_1 altered nearly in parallel with each other. In general, however, the shape of the flow curve was nearly the same in hyperaemia as in the normal animal, the only difference being that the mean flow level was raised in the latter. This can be seen in the records presented by Randall & Horvath (1953), and in those illustrated in Fig. 1.

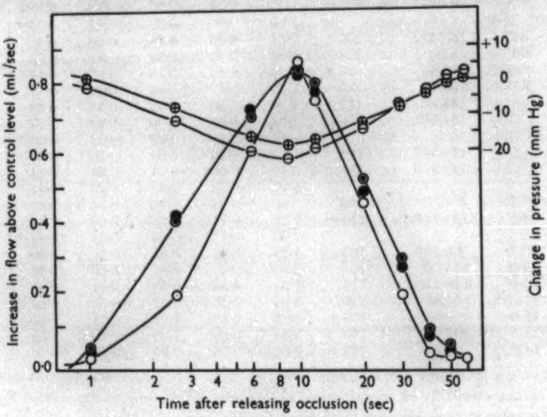

Fig. 3. Changes in carotid blood flow and pressure of a dog after a 5 min occlusion of the artery. ○, systolic spike of flow, P_1; ⊙, prediastolic dip of flow, V_1; ●, diastolic peak of flow, P_2; ⊕, maximum blood pressure; ⊖, minimum blood pressure. Negative values show decrease in the blood pressure below control level. Semi-log. scale.

DISCUSSION

The observations reported here on carotid and femoral flow in dogs agree with those of Shipley *et al.* (1943) and of Pritchard, Gregg, Shipley & Weisberger (1943) in their general trend. According to them the back flow is consistently found in the normal femoral, and frequently present in the carotid patterns. The magnitude of mean flow level or of the principal oscillations in the flow pulse waves found in the records presented in their papers is summarized in

Table 4. These values were obtained on dogs weighing 11–13·5 kg. Our results on dogs are compatible with those of Shipley and his colleagues, except that we have not found the negative flow component in the carotid artery so frequently as they stated. Their records also showed a parallelism between the mean rate of flow and the height of the diastolic peak (P_2), which agrees with our findings set out above.

Table 4. Values (ml./sec) from the pulsatile flow curves published by Shipley *et al.* (1943) and Pritchard *et al.* (1943) and recorded in the carotid and femoral arteries of dogs with an orifice meter. The points chosen correspond to those from our own results and the two values in each column represent minimum (left) and maximum (right) values in the series

	P_1		V_1		P_2		V_2		Mean	
Carotid flow	3·0	5·0	−0·3	2·5	1·5	3·0	1·0	2·0	1·0	3·0
Femoral flow*	2·5	3·0	−0·8	−1·5	0·3	1·5	0	1·0	0·5	1·5

* One record of femoral flow (Shipley *et al.* 1943) shows a very large difference between P_1 and V_1 (values about 6 and −2 ml./sec, respectively), and is not included.

The relative order in the heights of V_1 and V_2 seems also to agree with their results. Shipley *et al.* (1943) made the following statement: 'The striking feature which allows a differentiation of all the curves is the variability of the early diastolic rate of flow with respect to the presystolic rate. A comparison of this relationship with that of the corresponding early diastolic and presystolic points on the pressure curve reveals the superior mesenteric, renal, hepatic, common carotid, axillary and femoral patterns to have, in this respect, a progressively graded dissimilarity to their respective pressure curves.' This fact is, so far as the carotid and femoral patterns are concerned, exactly identical with that stated above in Results (p. 211). Such a feature is also recognized in the results of Richards & Williams (1953), in which the negative-flow component is entirely absent. It may be said, therefore, that the carotid and femoral flows at least, and all peripheral arterial flows probably, have their respective typical patterns determined by their anatomical and hydrodynamic characteristics. The differences in pattern are most marked in the early diastolic part of the flow pulse contours, and are more important than merely determining whether a back-flow component is actually present or not in the femoral flow.

Concerning this latter point, Shipley *et al.* (1943) and McDonald (1955) have maintained that negative flow is consistently present in the normal femoral flow and have cast suspicion on the fidelity of electromagnetic flowmeters, because flows recorded by this method (Richardson, Denison & Green, 1952; Randall & Horvath, 1953; Richards & Williams, 1953) failed to show a back-flow component. Inouye *et al.* (1955) have already discussed this problem and demonstrated its fidelity. The data presented here also support their view. The reason why other investigators who have employed the electromagnetic method failed to record a back-flow component in the femoral flow pattern is not apparent. Reactive hyperaemia as a result of the operations necessary for flow recording might play some part in this respect. If the recording is of short duration, after even a brief closure of blood vessels as employed by Richards & Williams (1953) there is the risk of its giving a relatively high flow level without a negative-flow component.

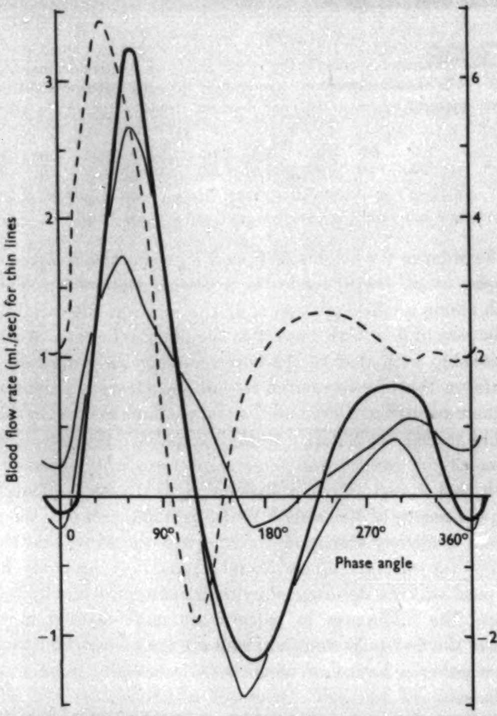

Fig. 4. Comparison of femoral flow curves obtained by various methods. Thick line, from Fig. 8, McDonald (1955) (ordinate on right); broken line, from Fig. 2, Shipley *et al.* (1943), (arbitrary ordinate scale); thin lines, examples of our records with a large and small back-flow component (ordinates on left).

In this connexion the fact mentioned in Results (p. 210) should be taken into consideration. As was pointed out by Inouye *et al.* (1955), a back flow of the same order in its magnitude as that reported by Shipley *et al.* (1943) or by Pritchard *et al.* (1943) can be obtained from the femoral patterns of Richards & Williams (1953) if the zero reference level is corrected so as to make the mean flow level similar to that of the former investigators, or that reported here. In other words, assuming the views of Shipley and his co-workers or of McDonald to be valid, such a correction seems to be necessary. This may be attributed to the instability of the electromagnetic method. In fact there are occasions when instability of unknown origin occurred in our instrument after recording was started. But the necessity of correction for the base line cannot be solely attributed to the instability of flowmeters, because the zero reference level has been checked by clamping the artery before and after its recording, confirming that these two zero levels coincided with each other and a drift never occurred. In the observations of Richards & Williams (1953), the same was the case. On the other hand, the fact that in our experiments correction of base line seems desirable only for some of the anaesthetized dogs, but not for rabbits under local anaesthesia, appears to suggest that variability in the degree of anaesthesia is in part responsible. This cannot be its sole cause, however, and it remains an open question as to what factors make it necessary to introduce a correction of base line in order to obtain consistent data.

The records obtained by the orifice meter on dogs weighing 11–13·5 kg (Shipley *et al.* 1943) show variability of the mean flow level, and its correlation with body weight of animals appears not to be established, although these authors did not display all their data and a definite conclusion cannot be drawn from these records alone. Such a correlation seems, from the physiological point of view, highly probable and the variability may be explained to some extent by altered circulatory conditions due to variable degrees of anaesthesia or to the calibre of the orifice. Meanwhile, the systolic spike and early diastolic (or prediastolic) negative flow in the femoral patterns reported by McDonald (1955) are considerably higher than those reported here and by Shipley *et al.* (1943). Such a difference might be due, at least in part, to an effect of the high resistance of the orifice meter. In McDonald's cinematograph method, on the other hand, excessive rates of flow might be recorded, because the bubble was in the axial stream. We have no experience, however, in working with an orifice meter, or of high-speed cinematography, and these points still remain uncertain.

We have considered above only the size of the main positive and negative oscillations in the flow pulse wave and not their timing, since the latter would be mainly determined by the relative length of systole in one cardiac cycle, which is fairly variable, together with the heart rate. On the other hand, McDonald (1955) discussed the time course of femoral flow curves in comparison with those of pressure or pressure gradients. Hence a comparison of our records with those of other investigators (Fig. 4) shows that the phase relation of our results agrees fairly well with that obtained by other methods.

The effect of reactive hyperaemia on the shape of flow pulse is illustrated in Fig. 5. Since the heart rate was not altered and the phase angle of the systolic spike is chiefly determined by the systolic ejection of blood, it is easy to understand that hyperaemia did not affect the position of the systolic spike on the flow curve. It is noted, however, that the prediastolic dip V_1, is delayed slightly in its occurrence when the flow level is raised, and the higher the flow level the more the former delay. The increase in flow with a decrease in pressure (see Figs. 1 and 3) suggests a loss of resistance to the flow distal to the occluded artery, to which such change in the flow curve would be attributable. In fact, in the records presented by Pritchard *et al.* (1943), we can find a decrease in depth of V_1 and a slight delay in its occurrence after intra-arterial injection of nitroglycerine or aminophylline, which also cause an increase in the mean flow level with decrease in pressure. Using the electromagnetic flowmeter, such effects of vasodilator drugs (acetylcholine, pilocarpine, amyl nitrite

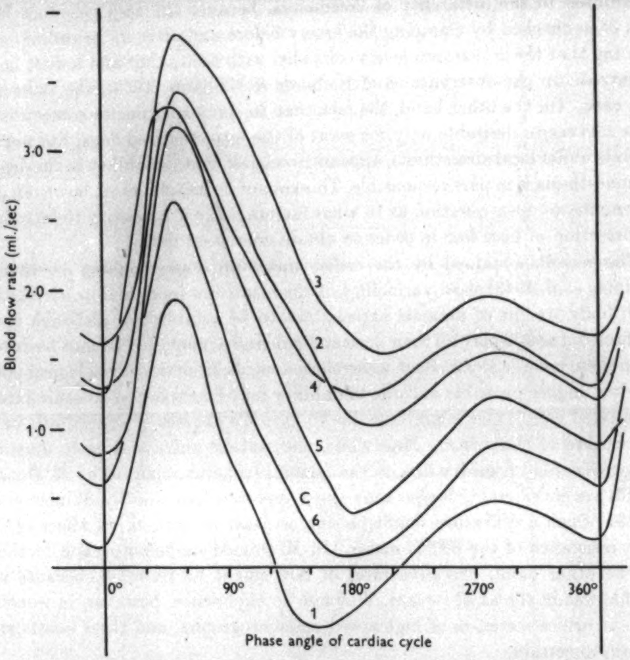

Fig. 5. An illustration of the effect of reactive hyperaemia on the form of flow pulsation. C, control flow pulse; 1, immediately after releasing occlusion; 2, after about 5 sec (rising phase); 3, after about 14 sec (peak); 4, after about 22 sec (falling phase); 5, after about 30 sec (nearly stationary level).

and aminophylline) have been confirmed in our laboratory (Inouye & Kosaka, unpublished). Reserving detailed description and discussion for a later paper, it might be stated that such a loss of peripheral resistance would cause a change in reflexion of pulse waves at the peripheral vascular bed distal to the occlusion, and alter the standing wave system along this arterial tree. Hence it is not so difficult to understand that the effect of reactive hyperaemia is chiefly reflected upon the early diastolic part of the flow curve, but not easy to explain it distinctly from the hydrodynamic point of view until more data are accumulated on the change in the flow pulse contour under varying conditions of the peripheral vascular bed.

SUMMARY

1. The flow pulse patterns in the carotid arteries of rabbits under local anaesthesia and that in the carotid and femoral arteries of anaesthetized dogs have been investigated by an electromagnetic flowmeter.

2. The carotid flow pattern rarely exhibits a back flow, but this is frequently observed in the early diastolic part of the femoral flow pattern. The recorded time course of flow pulse, and the size of its amplitude and mean level, agree fairly well with those previously obtained by other methods.

3. The mean flow level shows a fairly good correlation with the diastolic peak of flow pulse. A correlation between body size of experimental animals and the mean flow level or amplitude of flow pulse is also proved to some extent, especially for rabbits.

4. The effect of reactive hyperaemia following occlusion of an artery is shown. It is demonstrated that the form of flow pulsation is only slightly altered in its early diastolic phase, although back flow commonly disappears owing to the rise in mean flow.

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TEMPORARY NUTRITIONAL STRESS AND INFERTILITY IN FEMALE MICE

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Macfarlane, Pennycuick & Thrift (1957) have reviewed the literature on the factors causing embryonic mortality in rodents. These include a uniformly hot environment, underfeeding—particularly of protein and vitamins A, E and B group—removal of ovaries, an overdose of oestrogen, hypothyroidism, chronic hypoxia, excess of adrenal cortical hormones and injections of carbon tetrachloride and trypan blue. McClure (1958) reported the results of a preliminary experiment in mice in which infertility was associated with short periods of total starvation at or about the time of mating.

In this paper the results are given of a series of experiments designed to confirm these preliminary findings and to define the minimal stress required, the optimal time for the period of starvation and the constituent of the diet involved. The results of the preliminary experiment are not included.

METHODS

Randomly-bred white mice from a closed colony were successively bred for each separate experiment and fed on a standard pellet diet which had proved adequate for normal growth and reproduction of mice in the colony. These pellets were freely available except during the periods of starvation or other treatment. Water was always available. Air temperatures were maintained at 18–24° C. At the commencement of each experiment virgin female mice of about 2 months of age were placed singly in cages. On the next day one male was put with each female. Bucks were removed after 10 days. Vaginal smears for detection of oestrus were made each day while the bucks were present. Two hundred and sixty-two female mice in 19 groups were subjected to the following periods of complete starvation:

(*a*) a single 24 hr period starting at oestrus, at oestrus + 2 days, or at oestrus + 4 days;

(*b*) a single 48 hr period starting at oestrus, at oestrus + 2 days, at oestrus + 4 days, at oestrus + 8 days, or at oestrus + 16 days;

(*c*) two 48 hr periods, separated by 48 hr periods of feeding *ad lib.*, starting at oestrus, at oestrus + 2 days, at oestrus + 4 days, at oestrus + 8 days, or at oestrus + 12 days;

(*d*) three 48 hr periods separated by periods of 48 hr of feeding *ad lib.*, starting from the day of oestrus;

(*e*) three 48 hr periods during which the pellets were withheld, separated by 48 hr periods with pellets, starting from the day of oestrus. During the periods without pellets, the following foods were given *ad lib.* to mice in five groups, one type of food to each group: (1) heat-coagulated egg white, (2) heat-coagulated egg white plus raw rice starch, (3) raw rice starch, (4) raw rice starch plus sucrose, (5) a mineral solution containing (mg/100 ml.) NaCl 865, KCl 38, $CaCl_2 6H_2O$ 56, $MgSO_4 7H_2O$ 25. This was designed to supply the main minerals in approximately the same concentrations as they occur in plasma and replaced the drinking water.

Each experiment included control mice which were not starved or given alternate food. The total number of control female mice was 48. Results were assessed on the number of mice which littered to matings presumed to have occurred or detected by the presence of copulation plugs at the first oestrus after the males were introduced.

RESULTS

About half the possible matings were confirmed by the presence of a copulation plug, usually on the day and sometimes on the second day after the occurrence of oestrus. The number of mice littering and the mean litter number in each group are shown in Tables 1 and 2. In these tables the figures represent the total number of animals observed after both presumed and confirmed matings. The figures obtained only from the animals in which copulation plugs were observed show the same trend.

TABLE 1. The number of mice littering and the mean litter number (bold figures) in each group subjected to various periods of starvation after oestrus

Period of starvation	Number of days after oestrus that starvation was commenced					
	0	2	4	8	12	16
Nil (controls)	36/48 (75%) **7·5**	—	—	—	—	—
One 24 hr period	5/7 (71%) **7·4**	6/8 (75%) **8·3**	6/8 (75%) **8·8**	—	—	—
One 48 hr period	6/17 (35%) **6·0**	2/16 (13%) **7·0**	0/15 (0)	3/8 (38%) **7·3**	—	7/8 (88%) **6·0**
Two 48 hr periods separated by 48 hr periods of feeding *ad lib.*	0/13 (0)	0/16 (0)	0/15 (0)	1/16 (6%) **7·0**	7/8 (88%) **7·6**	—
Three 48 hr periods separated by 48 hr periods of feeding *ad lib.*	0/33 (0)	—	—	—	—	—

TABLE 2. Number of mice littering and the mean litter numbers (bold figures) after their complete pellet food had been replaced by protein, carbohydrates and/or minerals for three 48 hr periods after oestrus

Substitute food	No. littering	%	Mean no. in litter
Egg white	1/31	3	**6·0**
Egg white + starch	10/12	83	**6·1**
Starch	5/12	42	**6·3**
Starch + sucrose	5/11	45	**6·8**
Mineral solution	0/8	0	—

Controls have been considered as one group. In this 36 out of 48 (75%) littered, a figure in conformity with that obtained from previous experience of the mice in our colony. The effect of the different treatments relative to this can be briefly summarized as follows:

1. A single 24 hr period of starvation, starting at oestrus, at 2 or at 4 days later had no effect on the number littering.
2. A single 48 hr period of starvation reduced fertility markedly. Maximum effect (zero fertility) was recorded when starvation commenced at the fourth day. The proportion littering was reduced to half the normal when the treatment was applied at oestrus or at the eighth day after, but was normal when starvation commenced 16 days after oestrus.
3. Two separate 48 hr periods of starvation resulted in no mouse littering when treatment commenced at oestrus, at 2 days or at 4 days after. The effect was virtually the same in the 8-day group but disappeared completely with the 12-day group.
4. Three separate 48 hr periods of starvation commencing at oestrus resulted in complete failure to litter.
5. In the groups where special diets were given when normal food was withheld the littering rate was zero or low in the mineral-solution and egg-white groups, respectively, half the normal in the starch and starch + sucrose group, and normal in the egg-white + starch group.

The mean litter numbers in the groups ranged from 6 to 8·8, as shown in Tables 1 and 2. None of the treatments appeared to affect the number of mice born in each litter.

DISCUSSION

These results confirmed the preliminary finding that short periods of starvation after mating and during early pregnancy interfere with either fertilization or the maintenance of pregnancy. While there is no evidence to show the effect of starvation on fertilization, these experiments suggest strongly that starvation, delayed until after fertilization and even implantation should have occurred, must have caused embryonic death. The minimal period of complete starvation which produced zero fertility was the single 48 hr period from the fourth to the sixth day after oestrus. This period includes the time of implantation (Block, 1939). At times other than this, a 48 hr period of starvation was less effective. Two such periods separated by 48 hr periods of feeding *ad lib.* caused infertility in all mice when the starvation started from oestrus or up to 4 days later. When the start was delayed until 8 days after oestrus, starvation did not interfere with all pregnancies and when starting at 12 days after oestrus the fertility was normal.

There is much information in the literature dealing with long-term deficiencies of food constituents causing infertility. There is little information in the literature about short-term deficiencies having any effect. Severe underfeeding, starting from shortly after copulation and continuing through the normal period of pregnancy or until earlier death from starvation, inhibited pregnancy in 16/17 female rats (Barry, 1920). Nelson, Wright, Baird & Evans (1956) have shown that the administration of the folic acid antagonist, x-methyl pteroylglutamic acid, to rats for 36 hr starting on the eighth day of pregnancy caused the death of 65% of the embryos; administration for 48 hr caused death of all the embryos. Thiersch (1957*a*, *b*, *c*) has shown that other anti-metabolites given to rats during early gestation caused death of the foetuses.

The results reported in this paper suggest that embryonic death can be produced in mice simply by deprivation of all food for as short a term as 48 hr. Such treatment is unlikely to cause deficiency in the accepted sense of the term but is more likely to cause some type of physiological stress reaction. The observations of Robson & Sharaf (1952) and Macfarlane *et al.* (1957), that ACTH and cortisone in large doses interrupt pregnancy, may indicate the pathogenesis.

The evidence showing that egg white alone was not able to replace the complete pellet food, but egg white and starch did allow normal fertility suggests that the cause of the infertility was a deprivation of energy for short periods. Even though the fertility when only starch or starch plus sucrose was fed was not as high as normal, the evidence suggests that the carbohydrate fraction of food may be the important fraction involved. In experiments of this type, where one food is suddenly changed to another, it is difficult to ensure that the mice eat sufficient of the new food. It is possible that this may account for the only partially successful replacement of the pellets by starch or starch and sucrose.

SUMMARY

Complete starvation of mice for periods of 48 hr at various times during early pregnancy caused infertility. This effect was partly relieved by feeding carbohydrate during the periods when the normal food was withheld.

Acknowledgement is made to Miss S. Park for technical assistance.

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SINGLE UNIT ACTIVITY IN STRIATE CORTEX OF UNRESTRAINED CATS

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A beginning has recently been made in recording single neurone activity from animals with chronically implanted electrodes (Hubel, 1957*a*; Gusel'nikov, 1957; Ricci, Doane & Jasper, 1957; Strumwasser, 1958). These methods eliminate anaesthetics, paralysing drugs, brain-stem lesions, and other acute experimental procedures. They make it possible to record electrical events in the higher central nervous system with the animal in a normal state, and to correlate these electrical events with such variables as waking state, attention, learning, and motor activity.

The present paper describes a method for unit recording from the cortex of unanaesthetized, unrestrained cats, and presents some observations from the striate cortex. The objectives have been (1) to observe maintained unit activity under various conditions such as sleep and wakefulness, and (2) to find for each unit the natural stimuli which most effectively influence firing. Of 400 units observed, some 200 are presented here because of their common characteristics. Since there is reason to believe that the remaining 200 units were afferent fibres from the lateral geniculate nucleus, these will be described in a separate paper. A preliminary account of some of this work has been given elsewhere (Hubel, 1958).

METHODS

Unit recordings in unrestrained animals were made with micro-electrodes held in a positioner which was anchored rigidly to the skull during recordings, and removed between recordings. A rigidly implanted peg adapted from the design of Ricci *et al.* (1957) held the micropositioner at the time of recording (Text-fig. 1). The peg was made of the plastic Kel-F (fluorocarbon polymer made by Minnesota Mining and Manufacturing Company, St Paul, Minn.). It was hollow and was threaded outside and inside. The upper outside threads held the positioner; the lower ones served to screw the peg into the skull, and were made oversize to give a tight fit. The inside threads held a plastic plug which blocked the hole when recordings were not being made. To avoid sudden suction or pressure on the cortex the plug was fitted with a small machine screw which was taken out before the plug was removed or inserted.

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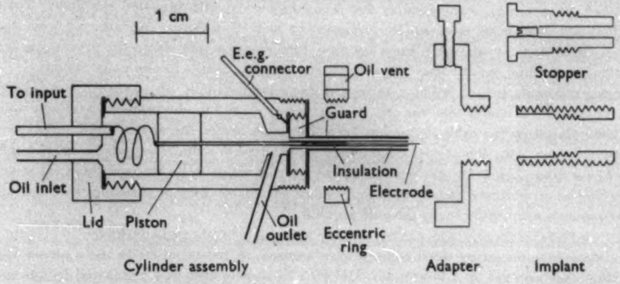
The peg was implanted under sterile conditions. The anaesthetized animal was placed in a stereotaxic apparatus, the site of implantation was determined, and an incision was made a small distance away. The skin was pulled aside to expose the bone at the point selected, and a hole was trephined and then threaded with a bottoming tap. In most operations the dura was then carefully removed. The peg was screwed in with a wrench until its base was level with the inner surface of the skull. The skin was pulled over the peg and a second incision was made just large enough to let out the peg, which was thus tightly surrounded by skin. The original incision was then closed separately. Animals prepared in this way rarely developed scalp infections. A stainless steel wire reference electrode was sutured to fascia and brought out at the nape of the neck. No recordings were made until at least twenty-four hours after operation.



Text-fig. 1. Diagram of hydraulic micropositioner and implant. Lid, piston and cylinder are made of Perspex; guard is pure silver, insulated inside with nylon sleeve; eccentric ring and adapter are aluminium alloy; implant and stopper are Kel-F. Rubber gaskets are shown between lid and cylinder, and above and below flange of guard.

The micropositioner was designed on a hydraulic principle so that adjustments could be made in the electrode's position without disturbing the animal (Text-fig. 1). The piston and cylinder were made of Perspex. In this transparent system air bubbles could be cleared and the piston's position seen. The piston held a concentric 22-gauge stainless steel needle whose lower end held the micro-electrode, and whose upper end was connected by a coiled wire to a contact pin in the lid of the cylinder. Mineral oil entering or leaving through a steel tube in the lid raised or lowered the piston. A polyethylene tube connected the oil inlet with a 1/4 c.c. syringe which was driven by a screw of pitch 1 mm per turn. One turn of the screw advanced the electrode about 100 μ.

Oil was kept in the area below the piston to prevent air from being sucked up between piston and cylinder. It escaped by the oil outlet into an open-ended polyethylene tube several feet long, which acted as a reservoir.

A silver tube was threaded into the base of the cylinder by a flange at its upper end. It served as a guard into which the micro-electrode could be retracted before attaching the micropositioner to the implanted peg. It also formed a monopolar surface electrode, making contact with the pool of cerebrospinal fluid near the cortex, but not touching the cortex. A fine nylon tube, lining the inside of the guard, insulated it from the steel needle and formed a smooth oil-tight fit with the needle. In this way the space below the piston was kept separate from the space above the cortex, which was thus effectively closed off from the atmosphere. This method of damping cortical pulsations was based on the closed chamber system described by Davies (1956).

The cylinder was attached to the Kel-F implant in a way which allowed variation in sites of successive penetrations. A cup-shaped aluminium adapter was threaded on the implant. In the cup was placed an eccentric or concentric ring which in turn held the cylinder. By rotating an eccentric ring in the cup, the electrode could be displaced up to 2 mm in any direction.

Except for a small amount of cerebrospinal fluid which covered the cortex, the peg was filled with mineral oil. Oil vents in the ring permitted excess oil to escape when the cylinder was attached.

The micro-electrodes, developed specifically for this type of work, were tungsten wires pointed by electropolishing and insulated with a vinyl lacquer (Hubel, 1957*b*). They were sturdy and sharp enough to penetrate leptomeninges up to several weeks after the implantation. Their tip diameter was less than $0{\cdot}5\,\mu$. In the visual cortex best results were obtained with electrodes coated to within $15\text{–}25\,\mu$ of the tip. These were made by dipping the pointed wire into lacquer with tip uppermost until all but the very tip, as seen under a dissecting microscope, was submerged.

Hardened stainless steel electrodes were used occasionally. They were pointed with direct current in a bath of concentrated *o*-phosphoric acid at 95° C, and coated by the procedure just described. In recording characteristics they seemed identical with tungsten electrodes. The main disadvantage of stainless steel was its comparative lack of stiffness. Marking by iron staining (Green, 1958) was not attempted since the procedure destroys the electrode, preventing further recording and ruling out the possibility of making multiple marks in one penetration.

During recording the cat lay on a table and was restrained only by a loosely fitting chest harness. The head was completely free (Pl. 1). Cats were selected to eliminate wild, unruly animals, and were placed on the table in harness for a few hours before the day of the implantation, to familiarize them with the surroundings. They soon adapted themselves, and would usually lie content for many hours. It was possible to record from single units for periods of up to several hours despite all but very violent head movements such as shaking or sneezing: paw-licking and face-washing movements usually gave no trouble.

Observations on sleeping and waking activity were made in complete darkness, to prevent light stimulation from occurring when animals were aroused. A hot room made the animals drowsy, and they were aroused by a brief noise. The state of wakefulness was monitored by the surface electrocorticogram.

For visual stimulation the cat faced a large white semicircular screen at a distance of eighteen inches. This could be lit diffusely by the room lights, or by a circular spot subtending an angle of 2° at the cat's eyes. The flashlight which made the spot was mounted so that swivelling it changed the setting of a potentiometer, deflecting one of the oscilloscope beams. This gave a record of the relative horizontal position of the spot. Usually a cat would follow the spot with head and eyes for only a few minutes after first seeing it. It would then stare at some point on the screen without moving head or eyes for several minutes at a time, long enough to permit localization and some exploration of a unit's receptive field.

Background illumination was generally such that the observer could see forms, but not colours. The stimulus spot measured about 1 cd/m^2 in brightness. The animal's pupils were not dilated, since one wished to keep the preparation as normal as possible. This had the advantage of preserving accommodation, and it was assumed that the retinal image was in focus. A given spot did not necessarily produce a retinal image of constant brightness, since the pupil diameter was not fixed and since eyelids and nictitating membranes were intact. This was probably not important for the qualitative observations of this study.

Conventional recording methods were used. The input stage was a cathode follower designed by Bak (1958). Since metal electrodes can become noisy when grid current is excessive, this was checked occasionally and kept to a minimum. The cathode follower was connected to the micropositioner by a flexible 8 in. (20 cm) cable whose shield was cathode-connected to reduce capacity to ground.

Pegs were implanted in thirty-five cats. They were placed over the striate cortex near Horsley-Clarke frontal plane zero, about 2 mm from the mid line. By the criteria of Talbot & Marshall

(1941) this should be close to the region where central vision is represented. The brains of all cats were examined grossly and histologically to confirm the area and to assess damage. In most brains grey matter appeared normal to Nissl stain, except for some round-cell infiltration which was confined to the leptomeninges, electrode tracks, and perivascular spaces.

The depths at which units were recorded were not accurately known because of the sharp curvature of the lateral gyrus, dimpling of the surface of the brain at the time of penetration, and difficulty, due to overlying cerebrospinal fluid, in knowing when the electrode made contact with the surface of the cortex. However, one could be sure that most units were less than 2 mm deep—the approximate thickness of the cortical grey matter—since the total distance travelled by the electrode was usually no more than this.

In six cats electrolytic lesions were made by passing 5 μA for 5 sec through the electode tip (electrode negative). Brains were perfused with saline followed by 10% formalin within 12 hr of making lesions. Serial paraffin sections 15 μ thick were stained with cresyl violet. Lesions were of remarkably uniform size and configuration (Pl. 2), and consisted of a dark-staining core about 35 μ in diameter surrounded by a pale halo. In larger lesions there was a clear area in the centre. The size of the halo was 100–200 μ in diameter, and was a great help in finding the lesion in a large number of serial sections.

When lesions were made in several closely spaced penetrations it became necessary to distinguish one penetration from the other. This was done by making a different number of lesions along each track, using the number of lesions to identify the penetration. Microscopic examination of electrolytic lesions such as that shown in Pl. 2 further supported the conclusion that most records were from the grey matter of lateral gyrus.

Since these electrodes can record from single myelinated fibres (Hubel, 1957*b*), it is not certain that all records were obtained from cell bodies; some may have been from axons of cortical cells or from afferent fibres. Electrophysiologically it is not easy to distinguish cell-body from axon records. Although some uncertainty remains in this respect, it is believed that most records were from cell bodies.

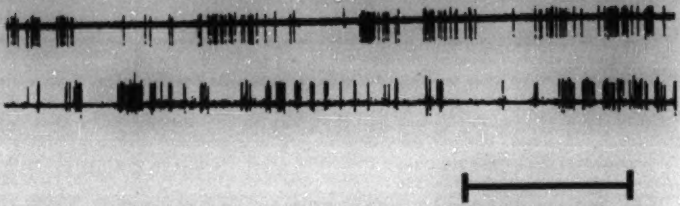
RESULTS

Background activity

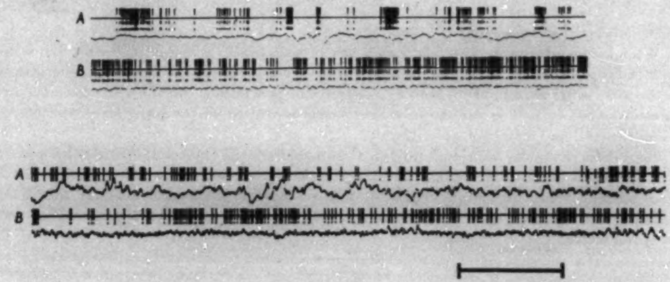
In the unrestrained preparation most units showed activity in the absence of intentional stimulation on the part of the observer. As the cat looked about, spurts and pauses in firing were seen to accompany eye movements. When the eyes were closed either passively by the observer or by the cat, firing usually persisted, although it was generally less active. Even when the room was made completely dark most units continued to fire.

Cortical unit discharges were very irregular. In the more active units firing often occurred in bursts separated by silent intervals (Text-figs. 2–4). Occasionally bursts recurred rhythmically for short periods, as is seen in Text-fig. 2. Even in units in which grouping was not obvious, firing was not nearly as regular as in other systems such as spinal motoneurones and peripheral stretch receptors.

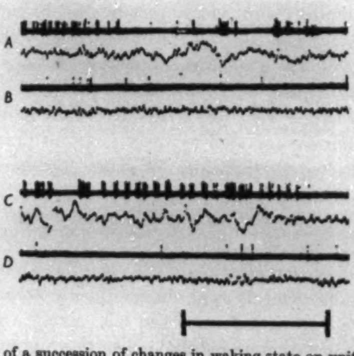
Satisfactory studies comparing activity in sleeping and waking states were made in twelve units. One typical change during arousal is illustrated in Text-fig. 3. Bursts were either smoothed out, giving a marked increase in regularity of firing, or they were reduced in length while occurring more often. In either case there was no obvious change in the over-all rate of firing.



Text-fig. 2. Two examples of cortical unit activity. Positive downwards, time constant 0·5 msec; time marker, 1 sec.



Text-fig. 3. Two examples of effects of arousal on cortical unit activity. For each unit *A* shows a record during sleep, and *B* a record after arousal. Upper beam, micro-electrode recording; lower beam, surface electrocorticogram; animal in complete darkness. Time marker, 1 sec.



Text-fig. 4. Effect of a succession of changes in waking state on unit firing: *A*, animal asleep; *B*, awake; *C*, asleep; *D*, awake. Beams as in Text-fig. 3; time marker, 1 sec.

In four units arousal affected firing in a different manner; a sharp reduction in rate occurred, and persisted as long as the animal was kept awake (Text-fig. 4). Brisk firing in rhythmic groups returned each time the animal resumed sleeping. During waking periods the few spikes that remained showed that the unit had not been lost.

Responses to visual stimuli

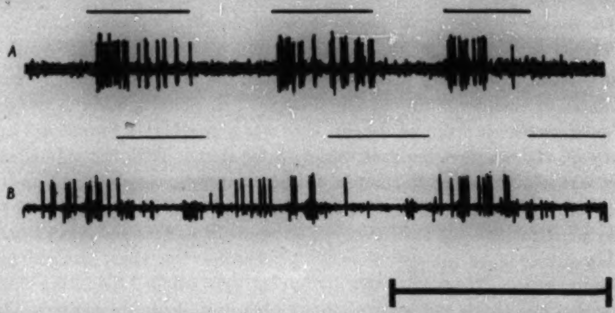
Changes in background illumination. It was found early in this study that most cortical units, other than afferent fibres, did not give clear or consistent responses to changes in diffuse illumination, that is, to lighting or darkening the entire screen in front of the cat. This was true whether the cat was awake or asleep, and whether the eyes were open or closed. With the animal awake there was seldom a clear response at the moment of turning the light on or off; while the light was on irregularities were sometimes introduced but these could usually be related to head or eye movements, and did not occur when the cat lay still or when the eyes were closed. During sleep there was also generally no response. There was little doubt that light reached the retina through the closed lids, since the same stimulus with eyes closed generally produced marked responses in lateral geniculate or optic tract neurones.

Units which were unresponsive to diffuse illumination could be strongly influenced by more specific visual stimuli. For instance, waving a hand in front of the cat made many units respond briskly, provided the movements were made in a particular and often very sharply restricted area of the visual field. It was concluded that these units were not concerned with registering changes in diffuse retinal illumination, but that they nevertheless played an active part in vision.

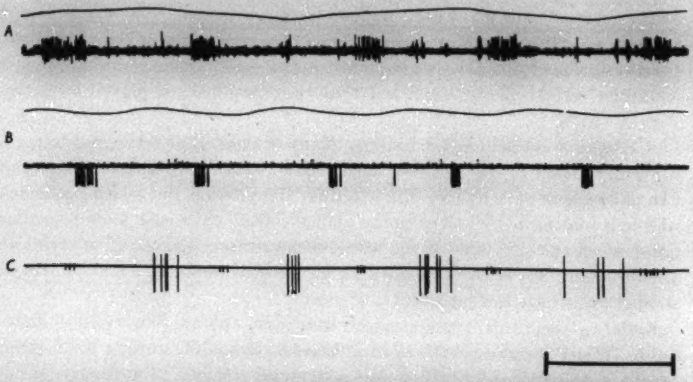
Responses to stationary light sources. Many of these units responded to turning a 2° spot on or off in a restricted region. Text-fig. 5 shows examples of two units which gave no response to diffuse illumination, but which responded well to a spot properly placed in the animal's field. With both units responses failed when the spot was moved about 2° away from the sensitive region in any direction. For most units the area from which responses could be evoked seemed to be near the cat's centre of gaze.

Both 'on'- and 'off'-type responses were seen, and are illustrated in Text-fig. 5. When turning on the spot produced discharges, turning it off often caused a transient suppression of background activity. Conversely, when illumination suppressed activity an 'off' discharge usually occurred. The phenomenon of reversal of a unit's response type—replacement of 'on'-type responses by 'off'- and vice versa—was not seen in this series. Each unit responded in its characteristic way when the spot was correctly placed, or gave no response at all (but see Discussion, p. 235).

Responses to movement. In all units a horizontally moving light source was at least as effective as a stationary one. Some responded each time the spot crossed the sensitive region, regardless of the direction of movement. In the example of Text-fig. 6*A* activation was equal for the two directions. However, in most units there was a difference in the size of the response, depending on whether the spot was moved from left to right or from right to



Text-fig. 5. Examples of responses of two different units to an appropriately placed 2° spot of light. *A*, 'on'-type response; *B*, 'off'-type response. Upper line indicates when spot is on; time marker, 1 sec.



Text-fig. 6. Three recordings of unit responses to horizontal movement. In *A* and *B* a 2° spot of light is shone on a screen: unit *A* fires each time the spot crosses the sensitive region, regardless of direction; *B* responds only when the spot crosses from left to right; downward deflexion of upper beam indicates movement of spot to right. In record *C* two units recorded simultaneously respond to to-and-fro hand movements of about 8 in. (20 cm), at a distance of 3 ft. (91 cm) from the cat; the experimenter's hand is illuminated by a light source behind the cat. Small spikes occur in response to movement to cat's right; large ones to cat's left. Time marker, 1 sec.

left. Often this difference was marked, and, in fact, many units responded consistently to movement in one horizontal direction but gave no response at all to the opposite movement. In the unit of Text-fig. 6*B*, for example, movement to the right gave a consistent response whereas movement to the left gave none. Units of this type often could not be activated by turning on or off a stationary spot.

The area of the cat's visual field over which movement was effective seemed to vary greatly in extent from unit to unit. Some gave a brief burst as the sensitive region was crossed; in others a continuous discharge was maintained as the spot traversed many degrees of field. Four units were activated by movement over a large part—perhaps 20°–30° or more— of the horizontal meridian (Hubel, 1958, fig. 15). In units of this type 'blinking' a stationary spot was always ineffective. The rate of movement was apparently not at all critical, and movements which were barely perceptible to the observer usually gave a strong steady discharge.

The two types of unit, one responding to leftward, the other to rightward movement, were found in one hemisphere, and were often seen in a single penetration. On at least three occasions two units which responded to opposing directions of movement were recorded simultaneously. In Text-fig. 6*C* neighbouring units responded reciprocally to to-and-fro hand movements. The experimenter stood about 3 ft. (91 cm) from the cat, and the excursions were about 8 in. It was thus clear that units with directionally opposite responses were to some extent intermixed. Coincidences of the type shown in Text-fig. 6*C* were rare, however. When single unit spikes were seen and heard against a background of many unresolved units, a common finding was that the isolated unit and the background units responded in a similar way.

DISCUSSION

In this paper two aspects of unit activity in visual cortex have been considered; background firing, including effects of sleep and arousal, and responses to several types of visual stimuli. Only a start has been made in either direction but a superficial survey may indicate areas for further effort.

The importance of chronic micro-electrode techniques in a study of background activity patterns and arousal effects hardly needs stressing, since the object was to make observations in *natural* sleep and waking states. Chronic techniques were also useful in studies of visual mechanisms. For instance, the observation that certain units did not respond to changes in background light would surely have lost much of its force in an acute experiment, since there would have been a suspicion that any one of several factors—anaesthetics, paralysing drugs, brain-stem section, low blood pressure, low temperature—contributed to the unresponsiveness. On the other hand, it seems likely that at least some of the responses described will be reproduced in the future by acute experimental methods; if so, detailed studies will be made easier and more precise by increased control over stimulation parameters and by even greater recording stability.

The existence of a maintained background activity at cortical levels is not surprising, since similar activity has been described in other parts of the mammalian nervous system. In particular, the maintained activity in the retina observed by Granit (1947) and by Kuffler (1953) may be pertinent, although at present we cannot say whether such activity is necessary for sustaining cortical background firing. Interrupting the optic nerves while recording from the cortex would help to settle this question.

The role of cortical maintained activity is unknown, but we may suppose that it provides a greater variety of response possibilities. Units can be inhibited as well as activated, and their firing patterns can be changed.

It is interesting to compare patterns of firing of cortical units with those of retinal ganglion cells, described by Kuffler, FitzHugh & Barlow (1957). It would seem that the grouped firing seen in so many cortical neurones occurs in ganglion cells only when they are abnormal or when the animal is deeply anaesthetized. The fact that in the cortex arousal could at times lessen or abolish this grouping is the main argument that grouping is a natural phenomenon, and is not necessarily a result of injury.

Two types of arousal effects were observed. The more usual, a tendency to smoothing out of bursts, resembles effects seen by Verzeano & Calma (1954) in the cortex and thalamus of curarized or Nembutalized cats. On the other hand, the four units in which arousal was linked with a marked decrease in firing recall the findings of Whitlock, Arduini & Moruzzi (1953), who observed suppression of firing in pyramidal tract fibres following arousal by reticular stimulation. Saito, Maekawa, Takenaka & Kasamatsu (1957) have reported similar suppression in the visual cortex of unanaesthetized, acute cat preparations aroused by electrical peripheral sensory and mid-brain reticular stimulation. It is interesting that in the first two studies rhythmic discharges were synchronized with sleep spindle activity. In the present work true spindles were seldom seen in the lateral gyrus at these levels of sleep, and efforts to relate individual surface sleep waves of less rhythmic type to unit bursts have as yet been disappointing. In Text-figs. 3 and 4, for example, there is no apparent correlation between the surface electrocorticogram and unit spikes.

The main object in studying cortical visual responses was to find the best types of natural stimuli for activating cortical units. Other workers (von Baumgarten & Jung, 1952; Jung & Baumgartner, 1955; Grützner, Grüsser & Baumgartner, 1958) have observed that a large group of cortical units do not respond to diffuse light stimulation. This was fully confirmed in the present study, where, indeed, only a minority of units were found to respond to diffuse light. However, a large number of cortical units which were unaffected by diffuse illumination responded well to a stationary 2° spot appropriately placed. The ineffectiveness of diffuse light is most easily explained by supposing that the response from the restricted region is inhibited by illumination of the surrounding visual field. That such antagonism between surround and centre of receptive fields occurs at the retinal ganglion-cell level in the cat is known from Kuffler's work (1952, 1953). Moreover, Barlow, FitzHugh & Kuffler (1957) have found that inclusion of the outer portion of a receptive field increases the threshold of a ganglion cell response. Wiesel & Brown (1958) have shown that such a rise in threshold is especially marked in ganglion cells of the cat's area centralis. This may be relevant to the present work, since recordings were presumably made from the corresponding cortical area.

In view of the suggestion of similarity in responses of cortical and retinal units, it is curious that changes in response type were not observed on stimulating peripheral parts of receptive fields. Specifically, 'on'-type responses were not observed to give way to 'off'-responses, and vice versa, when the spot was moved from the most sensitive area to closely adjacent regions. This has been seen in the great majority of ganglion cells in the light-adapted cat retina.

With the present light-stimulation methods, in the unrestrained preparation, it has been possible to change discharge types of optic tract neurones, in confirmation of the findings of Kuffler and his co-workers. Thus the method itself is capable of demonstrating the phenomenon. However, even at the ganglion-cell level the inhibitory surround is spread over a large region compared with the concentrated centre, and peripheral-type responses are best produced with annular stimuli. In the cortex the phenomenon may be reproducable only by using special stimuli confined to a large portion of the inhibitory region—whether annular or not one cannot say, since one has no idea of the shape of cortical receptive fields.

That some cortical elements respond to restricted light stimuli is consistent with the observations of Thompson, Woolsey & Talbot (1950) and Talbot & Marshall (1941), that localized cortical slow waves are evoked in response to restricted light stimuli in anaesthetized rabbits, cats, and monkeys. The present work gives little further information about detailed retinotopic representation, except for the finding that units in a part of the lateral gyrus (frontal zero) are usually driven by stimuli near the centre of gaze.

The most interesting results of these experiments came from the use of moving light sources. While responses to moving a spot in and across the sensitive region were expected (Hartline, 1940; Barlow, 1953), the greatly increased sensitivity of many units to a moving stimulus as opposed to a stationary one was surprising. The directional asymmetry of responses seen in so many units was completely unexpected.

One cannot at present be sure that these movement responses are elaborated at cortical rather than lower levels. Similar studies in other parts of the visual pathway would doubtless answer this. Such studies clearly are needed, for by observing responses to a wide variety of natural stimuli at each stage from retina to cortex we may hope to learn much about the function of each structure: we may yet find that something more than 're-representation' of topographic maps occurs at each stage.

The mechanism by which asymmetric movement responses arise is at present a matter for conjecture, since detailed information is lacking on the organization of cortical receptive fields. From the results presented it seems likely that at the cortical level receptive fields may vary according to the method of mapping, and that stationary spots and moving spots may give different results. By comparing fields mapped by the two methods we may gain some idea of how asymmetries in responses to movement originate. We may find, for example, that a cortical unit which responds differently to rightward and leftward movements has a stationary-spot receptive field in which the inhibitory and excitatory areas are specifically arranged, differing from the usual symmetry of retinal fields. With a knowledge of stationary-spot fields it may be possible to predict movement asymmetries. If, as this work suggests, some units do not respond at all to stationary spots, it may become necessary to use more elaborate methods, such as a pair of spots spaced a variable distance apart and timed so that one precedes and conditions the response to the other.

The observation that movement can activate a unit over a relatively large region of the visual field—of the order of 20° (5 mm on the retina) and perhaps more—suggests that convergence from a large retinal field must occur on some cortical units. This has already been suggested on anatomical grounds by O'Leary (1941). One must conclude that the striate cortex is not simply a retinotopically organized 'point-to-point' relay station along the visual pathway. The presence of convergence from large areas of retina would not, of course, exclude some sort of retinotopic representation, which, in fact, the present work tends to support. The cortex would seem to contain units with receptive fields—using the term in a broad sense to include moving stimuli—of widely differing extent.

Finally, one must for the time being be content to list several possible roles which these units responsive to movement may fulfil in visual mechanisms. They may enable moving objects in the cat's visual field to command more attention than stationary light sources. They may, on the other hand, be important for vision of stationary objects, since head and eye movements can produce movements of images across the retina. As a third possibility, they may, by projection to centres for head and eye movements, enable the organism to maintain fixation on a moving object. The last possibility is especially attractive, since it would fit well with the marked directional asymmetries which were so often observed. If one knew where axons of the units projected it might be easier to decide between these alternatives.

SUMMARY

A method is described for recording from single units in unanaesthetized, unrestrained cats. The following observations were made in a survey of a small region of the cat's striate cortex:

1. Most units showed firing in the absence of intentional stimuli, regardless of the waking state, and even with the animal in total darkness.
2. Cortical unit firing was generally very irregular. Grouped patterns were common, and groups often recurred rhythmically.
3. Two main arousal effects were seen: some units showed a smoothing out of grouped activity, with little change in rate of firing, others showed a marked decrease in firing rate.
4. Diffuse retinal illumination produced little or no response in most units.
5. Many units which were unresponsive to changes in background illumination responded briskly to a restricted light source. Both 'on'- and 'off'-type responses were seen.
6. The receptive field of most units was near the centre of gaze.
7. Units which were affected by stationary spots were also affected by a horizontally moving light source. Crossing the sensitive region produced discharges which were sometimes unequal for the two directions of horizontal movement. Many units responded to movement in one direction only. Some of these gave no response to a stationary spot. The region over which responses to movement could be evoked varied greatly from unit to unit, and ranged from a few degrees to about twenty.

I wish to express my gratitude to Drs M. G. F. Fuortes and R. Galambos for their continued advice and encouragement, and to Mr C. O. Henson for his unfailing technical assistance.

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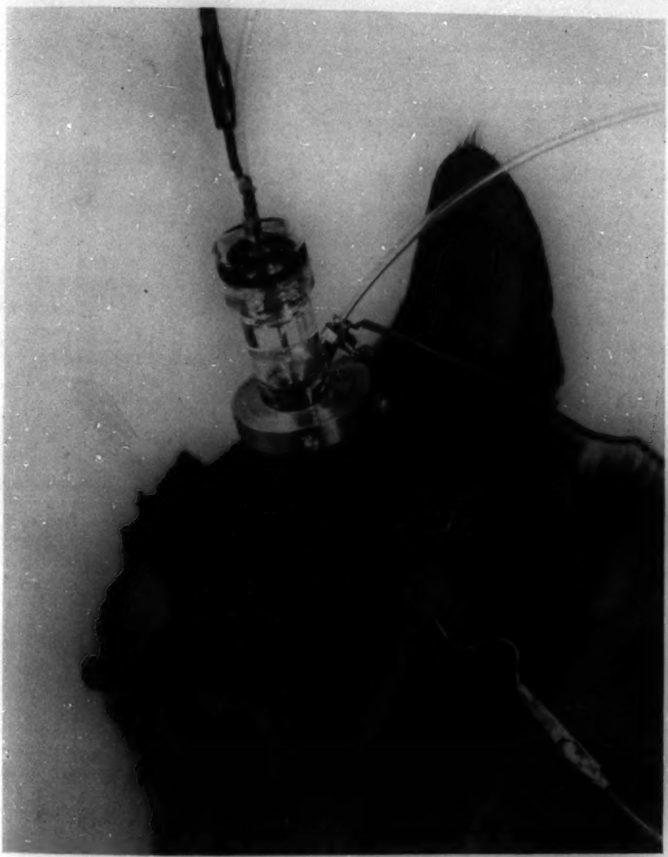
EXPLANATION OF PLATES

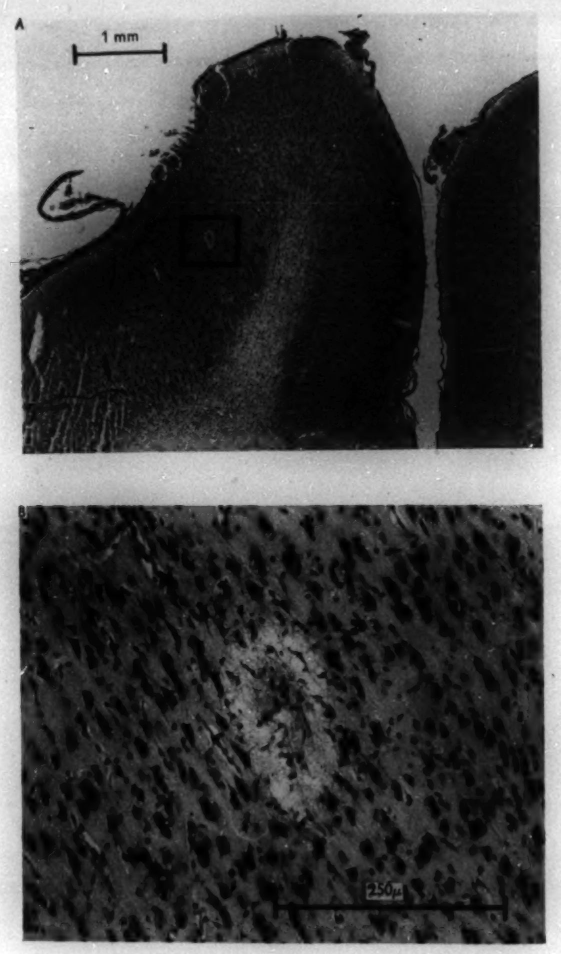
Plate 1

Photograph of cat with micropositioner in place for recording. Polyethylene tubes are attached to inlet and outlet vents, and connexions are made to the input terminal and to the e.e.g. connector. The implant is over the right lateral gyrus. The indifferent electrode is of stainless steel, sutured to the scalp.

Plate 2

Photomicrographs of Nissl-stained frontal section through an electrolytic lesion (in rectangle) made while recording from a cortical unit. *A*, low power; *B*, high power.




A
1 mm
B
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RESTORATION OF NEUROMUSCULAR TRANSMISSION IN SODIUM-FREE HYDRAZINIUM SOLUTION

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Neuromuscular transmission in frog nerve-sartorius preparations, completely blocked by soaking in a sodium-free sucrose solution, was found to be restored by immersion in a sodium-free hydrazinium solution (Koketsu & Nishi, 1958). The present paper describes results of experiments performed to clarify the mode of neuromuscular transmission in this solution.

METHODS

Preparations. Experiments were done mainly with nerve-sartorius preparations, but in some cases with iliofibularis muscles of frogs (*Rana pipiens*) at room temperature (21–24° C).

Solutions. The Ringer's solution consisted of (mM) NaCl 115·6, KCl 2, $CaCl_2$ 1·8, $NaHCO_3$ 2 (pH 6·8–7·0). The sodium-free sucrose solution used throughout consisted of (mM) sucrose 224, $KHCO_3$ 2, $CaCl_2$ 1·8 (pH 6·8–7·0). Hydrazine NH_2–NH_2 (Eastman) was used to prepare the sodium-free hydrazinium solution. The hydrazine solution (224 mM) was first adjusted to pH 6·5 with 224 mM-HCl, and was then made up to 2 mM-KCl and 1·8 mM-$CaCl_2$. The osmotic pressure of this solution, containing approximately 120 mM hydrazine, was practically identical with that of normal Ringer's solution; the freezing point of these two solutions was measured to be −0·465° C. This solution was mixed in various proportions with the 224 mM sucrose solution containing 2 mM-KCl and 1·8 mM-$CaCl_2$, for the purpose of making test solutions containing different amounts of hydrazine. Each test solution was prepared immediately before use, and was again adjusted exactly to pH 6·5 with HCl. Since hydrazine is a weak base and since only the hydrazinium ion is a substitute for sodium ion, the pH of the sodium-free hydrazinium solution should not be higher than 6·5–7·0 (cf. Lorente de Nó, Vidal & Larramendi, 1957). Flame photometry proved that the sodium ion concentration in the test solutions was below 0·01 mM.

Recording system. Isometric muscle responses to nerve stimulation or to directly applied acetylcholine (ACh) were recorded with a mechanical transducer (Grass), an amplifier and electroencephalograph (type D, Offner). Large platinum wires served as leads for extracellular electrical recording from whole tissue. Intracellular recordings were done according to the method of Fatt & Katz (1951). Intracellular potential changes were recorded with d.c. amplification except for spontaneous miniature end-plate potentials which were recorded with a.c. amplification. To measure the resting potential of the end-plate membrane and also the sensitivity of the end-plate membrane to directly applied ACh, the distribution of the surface potential of sartorius muscle was recorded by the experimental arrangement originally described by Fatt (1950) and later modified by Koketsu & Gerard (1956).

Procedure. Preparations were first tested for normal activity in Ringer's solution, and were then soaked in sodium-free sucrose solution (30 min unless otherwise specified) before immersion in a sodium-free hydrazinium solution.

RESULTS

Mechanical responses to nerve stimulation

Neuromuscular transmission in nerve-sartorius preparations was completely blocked by soaking in sodium-free sucrose solution for more than 20–30 min, and no mechanical responses resulted from supramaximal nerve stimulation. When these preparations were then immersed in hydrazinium solution, transmission was restored within 5–10 min, and mechanical responses similar to, or even more powerful than, normal ones were observed. Such restoration of transmission in the hydrazinium solution occurred regardless of the period of previous soaking in sucrose solution, complete restoration being observed even after 180 min soaking. A concentration of 80–90 mM hydrazinium was found to be most effective in restoring and maintaining function. All results herein described were obtained with this concentration. Transmission restored by the hydrazinium solution was not maintained indefinitely; blockage occurred gradually in preparations soaked for an extended time.

An example of the experimental results shown in Fig. 1 was obtained by using 85 mM hydrazine. Transmission, which was completely blocked by soaking in sucrose solution for 20–180 min, showed complete recovery within 10 min in hydrazinium. After 30–40 min transmission block recurred and was complete in about 60–70 min after immersion in the hydrazinium solution. Such late blockage occurs whether nerve stimulations are applied or not. It can in turn be reversed by re-immersion in Ringer's solution.

Electrical responses of muscle fibres

For preliminary observation, electrical responses of the whole sartorius muscle and the nerve twig innervating it were recorded simultaneously with the preparation soaked either in sucrose, or hydrazinium solutions. Figure 2 shows an example of results obtained in such experiments. The electrical activity of the nerve twig was not abolished in the sucrose solution; it only diminished slightly in 60–90 min, and did not disappear even after 180 min in the sucrose solution. This could be because the nerve sheath of the twig was not removed in this, or in any of these experiments. The muscle fibre electrical activity, however, disappeared completely within 20–30 min in sucrose solution. Soon after immersion in the hydrazinium solution, muscle electrical activity reappeared, as is shown in record 3 of Fig. 2. Some characteristic differences, however, were observed between the two electrical activities recorded in the Ringer's and in the hydrazinium solutions. First, the amplitude of the action potential in the hydrazinium solution was much smaller and less synchronized, compared to that in the Ringer's. Secondly, the muscle response latency was much longer in hydrazinium solution than in the Ringer's, suggesting that either the transmission delay was prolonged or the conduction velocity along the nerve fibre, particularly along its terminal endings, was slowed down.

Fig. 1. Isometric mechanical muscle responses produced by supramaximal nerve stimulations obtained from three different nerve-sartorius preparations. 10 nerve stimulations were applied at 1 sec intervals in each record. *A*: Record 1 was obtained in Ringer's solution. Record 2 shows that indirect responses were blocked completely 30 min after soaking in sucrose solution. The preparation was immersed in hydrazinium solution immediately after record 2, and records 3–12 were obtained 10, 20, 30, 35, 40, 45, 50, 55, 60 and 65 min thereafter. B : Record 1 was obtained in Ringer's solution. Record 2 shows that responses were blocked 60 min after soaking in sucrose solution. Record 3 was obtained 15 min after re-immersing the preparation in hydrazinium solution. C: Record 1 was obtained in Ringer's solution. Record 2 was obtained 20 min after soaking this preparation in sucrose solution. Record 3 was obtained 15 min after re-immersion in hydrazinium solution (note that the mechanical responses in record 3 are slightly facilitated compared with those of record 1).

Calibration shown in record 2 (A and C) shows 0·9 g deflexion; and records shown at the bottom are stimulation marks applied at 1 sec intervals; note that record C was obtained by use of a.c. amplifier.

Intracellular potential changes due to nerve stimulation. The resting potential of muscle fibres was slightly lowered in the hydrazinium solution, and there was a tendency for it to fall gradually during prolonged immersion (see Table 1). Muscle action potentials evoked by nerve stimulation were recorded intracellularly within 5–40 min after immersion in hydrazinium solution. Two examples of typical action potentials are shown in records 2 and 3 of Fig. 3, as compared to a control record obtained in Ringer's solution (record 1).

The action potential in the hydrazinium solution resembled that in the Ringer's with the exception that: (1) the spike potentials were practically equal in amplitude to the values of the resting potentials, and did not overshoot and (2) the duration of the spikes was slightly prolonged, with slow rising and falling phases. The muscle spike obtained from the vicinity of the end-plate arose out of the end-plate potential and formed a step on the rising phase (see record 3 of Fig. 3). The spike potential in the hydrazinium solution was followed by a marked negative after-potential similar to that in the Ringer's solution.

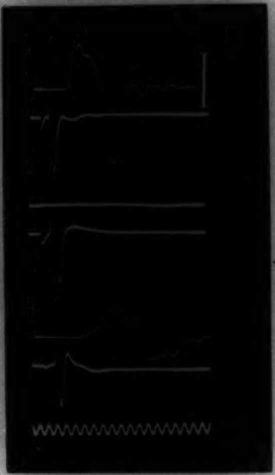

Fig. 2. Each record shows action potentials recorded from the whole sartorius muscle (upper traces) and from the nerve twig innervating it. Single shocks were applied to the nerve. All records were obtained from one preparation; records 1, 2, and 3 were made with the preparation first in Ringer's solution, then 30 min in sucrose solution, and finally 15 min in hydrazinium solution. The muscle action potential disappeared completely in sucrose solution, and recovered in hydrazinium solution, the action potential of the nerve twig was not appreciably affected. Note the prolonged latency and dispersion in the muscle action potential in record 3. Calibration, 10 mV; time marker, 1 msec.

Intracellular potential changes resulting from intracellular stimulation. Two examples of membrane potential changes induced by intracellular stimulation in hydrazinium solution are shown in records 5 and 6 of Fig. 3, as compared to a control record obtained in Ringer's solution (record 4). In comparison to the spike potentials in the Ringer's, those in hydrazinium solution were of smaller amplitude and longer duration, as was also seen in the indirect responses. A significant change in the hydrazinium solution was a threshold increase (critical depolarization) for the initiation of spike potentials. The mean value of the critical membrane potential for spike initiation, obtained from six different fibres of a preparation immersed in Ringer's solution, was 49 mV, and was 38·6 mV for six different fibres of the same preparation soaked in hydrazinium solution for 10–60 min.

Direct muscle responses could be obtained even after more than 60 min immersion in hydrazinium solution (see record 6 of Fig. 3); those accompanied by a powerful muscle contraction could be easily recorded even after immersion for 180 min. The threshold for the direct responses, however, increased considerably after such extended immersion.

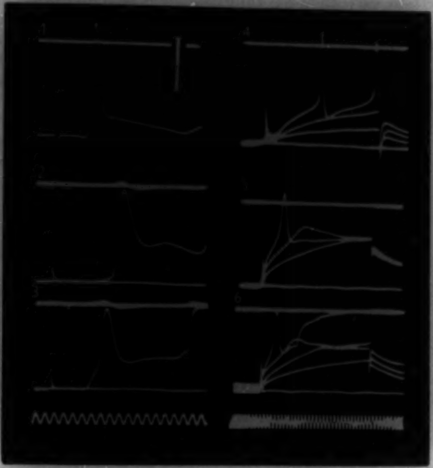

Fig. 3. Intracellularly recorded muscle fibre action potentials evoked by nerve stimulation (records 1–3) and by direct intracellular stimulation (records 4–6). Records 1 and 4 are potential changes in Ringer's solution, the other records are obtained in hydrazinium solution. Records 2, 3 and 5 were obtained between 5 and 30 min after, and record 6 about 80 min after the preparations were immersed in hydrazinium solution. In indirect responses potential changes were evoked by single nerve stimuli, and in direct responses action and electrotonic potential changes were evoked by a suprathreshold and two or three subthreshold stimulations. Local responses seen in records 5 and 6 were produced by just-subthreshold stimulations. The later time course of the action potential was disturbed in several records by muscle contractions. Calibration, 50 mV; time marker, 1 msec.

Electrical properties of muscle fibres

The electrical properties of resting muscle fibres in hydrazinium or Ringer's solutions were compared. Two micro-electrodes were inserted close together into a muscle fibre. By using one as a stimulating and the other as a recording electrode it was possible to calculate the effective resistance and time constant of the membrane from the electrotonic potentials produced by subthreshold, rectangular, direct currents (Koketsu & Nishi, 1957). It is clear that there were no appreciable differences between effective membrane resistance or membrane time constant in the Ringer's and hydrazinium solutions. If the assumption is made that the fibres measured in the Ringer's and those measured in the hydrazinium solution were the same in size and specific internal resistance, then it may be concluded that there was also no appreciable difference in the specific membrane resistances (Koketsu & Nishi, 1957). This suggests that ionic permeability of the resting muscle membrane was essentially the same in hydrazinium as in Ringer's solution.

TABLE 1. The resting potential and electrical properties of a sartorius muscle in Ringer's and in hydrazinium solutions. Measurements were taken from six different large fibres in one muscle; first in Ringer's solution, secondly from 10 to 30 min and finally from 90 to 120 min after immersion in hydrazinium

Fibre	Resting potential (mV)	Effective membrane resistance (kΩ)	Membrane time constant (msec)
	Soaked in Ringer's solution		
1	100	135	12
2	100	145	14
3	100	135	15
4	95	110	14
5	100	120	13
6	100	150	12
Mean	99	130	13·3
	Soaked in hydrazinium solution for 10–30 min		
1	88	112	15
2	86·5	114	10
3	96·5	180	10
4	90	114	14
5	87	117	14
6	88	209	15
Mean	89·3	141	13
	Soaked in hydrazinium solution for 90–120 min		
1	87	93	10
2	72	243	12
3	73·5	145	12
4	78	102	8
5	77·5	108	15
6	72	115	12
Mean	81·1	134	11·5

End-plate potential

End-plate potentials could be recorded from preparations immersed in a hydrazinium solution containing $3\text{–}5 \times 10^{-6}$ g/ml. tubocurarine chloride. A typical example of such potentials is shown in record 2 of Fig. 4, where it is compared with a record obtained in Ringer's solution. End-plate potentials obtained by focal recording (recording from the vicinity of the end-plate) showed a slower rising phase and somewhat rounded peak as compared with controls in Ringer's solution. Furthermore, the falling phase considered as a passive decay (Fatt & Katz, 1951) apparently shows a longer half-time for decay in hydrazinium solution.

Detection of end-plate potentials became very difficult if immersion in hydrazinium solution was longer than 30 min, although the muscle fibres still responded to nerve stimulation. At this stage the end-plate potentials did not diminish gradually but were abolished abruptly during repetitive nerve stimulation. This suggests that the blockage of transmission was not due to a gradual depression of the excitability of the pre- or post-synaptic membrane, but due to blockage of nerve conduction, which probably occurred at the terminal axon. The spontaneous miniature end-plate potential (Fatt & Katz, 1952*a*) was also recorded from uncurarized preparations (see record 3 of Fig. 4). No appreciable differences were observed in the frequency and the amplitude of these potentials obtained in hydrazinium or in Ringer's solutions.

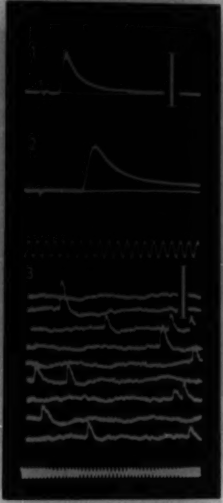

Fig. 4. Typical end-plate potentials recorded intracellularly at the vicinity of the junctional region of a curarized nerve-muscle preparation in Ringer's (record 1) and in hydrazinium solutions (record 2). Note the longer latency and slower rising and falling phases in record 2. Record 3 shows spontaneous miniature end-plate potentials recorded from an uncurarized preparation in hydrazinium solution; successive records were taken by shifting the trace vertically on the face of the oscilloscope. An a.c. amplifier with high amplification was used for this recording. Calibration, 20 mV (records 1 and 2) and 5 mV (record 3); time marker, 1 msec.

Resting potential of end-plate membrane

The resting potential of the end-plate membrane was measured by recording the distribution of the surface potential of the sartorius muscle (Fatt, 1950; Koketsu & Gerard, 1956). A uniform potential (variation did not exceed 1 mV) could be obtained from two points on the surface of an uninjured sartorius muscle in Ringer's solution. When the preparations were soaked in the sucrose solution for 30–60 min these potential differences were usually produced in an irregular manner along the muscle surface, as described by Fatt (1950). However, when these preparations were immersed in hydrazinium solution such unsteady potential differences disappeared and a uniform potential, similar to that observed in the Ringer's solution, was obtained for 10–180 min. This indicates that no appreciable changes were produced in the resting potential of the end-plate membrane, although a slight depolarization probably occurred in parallel with that observed in the resting potential of the whole muscle fibre (see Table 1).

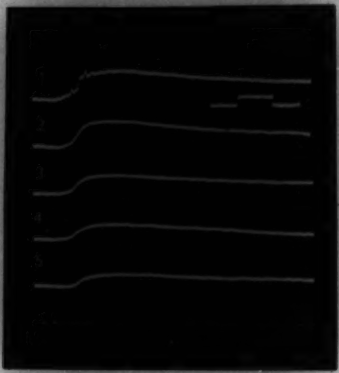

Fig. 5. Isometric mechanical responses of the iliofibularis muscle produced by the application of ACh (5×10^{-8} g/ml.) Record 1 is in Ringer's solution. Records 2–5 were obtained 15, 60, 90 and 180 min after immersion in hydrazinium solution. After taking record 1 the preparation was washed repeatedly in Ringer's solution, and after records 2–5 in hydrazinium solution. Calibration, 0·45 g; time marker, 2 sec.

Sensitivity of the end-plate membrane to applied ACh

The tonic mechanical responses of the muscle to directly applied ACh of different concentrations were studied by using the iliofibularis muscle. Visible muscle concentrations were observed on adding ACh of concentrations higher than 1×10^{-8} g/ml. to the Ringer's solution. After soaking in sucrose solution for more than 30 min, however, no visible contractions were produced, even with ACh at concentrations of 1×10^{-5} g/ml. Upon immersion in hydrazinium solution ACh sensitivity and contractions immediately returned. This recovered sensitivity was well maintained, showing only slight diminution after immersion for 180 min in hydrazinium solution. An example of the contractile response to ACh 5×10^{-5} g/ml., which lasted for 15–180 min in hydrazinium solution, is demonstrated in Fig. 5. In this figure, the amplitude of the contraction curve obtained 180 min after immersion in the hydrazinium solution was approximately half of that observed in Ringer's. Such reduction of the contraction, however, usually resulted also in the Ringer's solution, if ACh was applied repeatedly to the preparation.

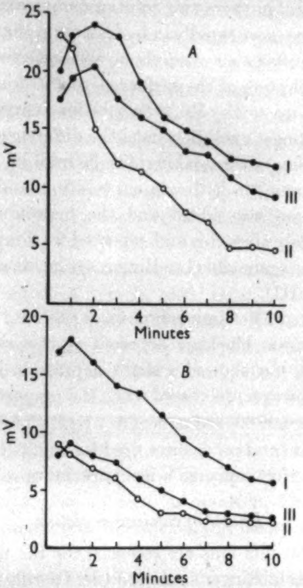

Fig. 6. Sensitivity of the end-plate membrane to directly applied ACh (5×10^{-5} g/ml.). The distribution of the surface potential of the sartorius muscle was measured and the maximum value of the depolarization (ordinate) was plotted against time (abscissa) after application of ACh. Diagrams *A* and *B* were obtained from two different preparations. Measurements were made first in Ringer's solution (curve I), secondly in hydrazinium (curve II) and finally in Ringer's (curve III). Curve II was obtained 15 (*A*) and 90 min (*B*) after immersion in hydrazinium solution. (For details see text.)

Sensitivity of the end-plate membrane to directly applied ACh was studied by measuring changes in the distribution of the surface potentials of the sartorius muscle (Fatt, 1950; Koketsu & Gerard, 1956). The maximum value of the end-plate depolarization was plotted every minute for 10 min after the application of ACh (5×10^{-5} g/ml.) in Ringer's or hydrazinium solution. The conductivity of hydrazinium solution (containing 80–90 mM hydrazine) was measured by use of a Wheatstone's bridge; the ratio of conductance of this solution and of normal Ringer's solution was found to be 0·71 : 1. Sensitivity was practically identical in these two solutions immediately after application of ACh, but it decreased more rapidly in hydrazinium than in Ringer's solution. Diagram *A* of Fig. 6 shows an example of this experimental series. In this experiment, the distribution of the surface potential was measured after ACh 5×10^{-5} g/ml. was added to the Ringer's solution (curve I). The preparation was then washed in Ringer's solution until the difference in the surface potential disappeared. Next, after soaking for 30 min in sucrose solution, the preparation was immersed in hydrazinium solution, and 15 min later ACh of the same concentration was added and the measurements were repeated (curve II). Immediately after this and repeated washing in Ringer's solution, ACh 5×10^{-5} g/ml. was again added to Ringer's solution and the depolarization was measured (curve III).

Sensitivity, measured after immersion for 30–60 min in hydrazinium solution (partial transmission blockage occurred at this stage), was practically normal in most cases, but showed a slight depression in some preparations. Sensitivity to ACh, however, decreased when the preparation was immersed in hydrazinium solution for 60–180 min. The results plotted in Fig. 6*B* were obtained from experimental procedures used for Fig. 6*A*, except that curve II was obtained 90 min after immersion in hydrazinium solution.

Effects of calcium depletion

It is known that calcium ions are essential for the maintenance of neuromuscular transmission in Ringer's solution (del Castillo & Stark, 1952; Fatt & Katz, 1952*b*). It was found that calcium ions are also essential in sodium-free hydrazinium solution. Neuromuscular transmission restored in hydrazinium solution was readily blocked when immersed in hydrazinium solution from which $CaCl_2$ was omitted. Transmission was not restored when the preparation was immersed in Ca-free hydrazinium solution immediately after soaking in sucrose solution.

The neuromuscular block during calcium deficiency is considered to be due mainly to a reduction in the ACh output from active motor nerve endings (del Castillo & Stark, 1952; Fatt & Katz, 1952*b*). The effect of calcium removal in hydrazinium solution, however, did not seem to be specific for activity of the motor terminal endings. As observed in frog spinal ganglion cell bodies (Koketsu, Cerf & Nishi, 1959*b*), whose excitability was easily eliminated if the calcium ion was absent from the sodium-free media, a number of muscle fibres lost excitability and no electrical responses were elicited by intracellular direct stimulation in Ca-free hydrazinium solution. The effect of calcium deficiency on the excitability of muscle fibres will be reported in detail in another paper.

DISCUSSION

During the course of the present experiments, it has been confirmed that the excitability of frog muscle fibres cannot be maintained when the external sodium is totally replaced by any one of several quaternary ammonium ions (choline, tetramethylammonium, tetraethylammonium, and tetrabutylammonium) (cf. Hagiwara & Watanabe, 1955). It has, however, been demonstrated that such excitability is maintained if hydrazinium ion is substituted for sodium ion. Thus, under favourable circumstances, frog muscle fibres are capable of maintaining excitability in the absence of external sodium. Crustacean muscle fibres have earlier been shown to maintain their excitability in media where sodium is wholly replaced by different quaternary ammonium ions (Fatt & Katz, 1953; Fatt & Ginsborg, 1958), and mammalian smooth-muscle fibres have been found to maintain their excitability in sodium-free choline solution (Burnstock & Straub, 1958; Daniel & Singh, 1958; W. Woodbury and M. Goto, personal communication). Similar behaviour has been reported for frog nerve (Lorente de Nó, 1949; Larramendi, Lorente de Nó & Vidal, 1956; Lorente de Nó *et al.* 1957; Deck, 1958; Lüttgau, 1958) and for mammalian nerve (Sugaya & Laget, 1958), and for frog spinal ganglion cell bodies (Koketsu, Cerf & Nishi, 1958*a*, *b*, 1959*a*, *b*). The generalization seems to emerge that excitability of various excitable membranes is maintained in media where the sodium ion is replaced by a specific onium ion possessing a favourable affinity for the membrane in question. The possible mechanism of electrical activity in sodium-free media will be fully discussed in another paper.

It is further demonstrated in the present experiment that neuromuscular transmission can be maintained in sodium-free solution, showing that excitability is maintained in the absence of the external sodium not only in the muscle conductile membrane but also in the muscle end-plate membrane. The excitability of the post-synaptic membrane is considered to be different from that of the conductile membrane. The mechanism of production of the end-plate potential has been described as a large, non-selective increase in ion permeability of the membrane, produced by a chemical transmitter (ACh) (Fatt & Katz, 1951). Fatt (1950) reported that ACh still evoked a depolarization of the end-plate membrane after sartorius muscles were immersed in sodium-free glucose or sucrose solution (1–3 hr); the maximum depolarization was about 50% of that occurring in the presence of sodium. According to more recent investigations, however, the depolarizing effect of ACh is reduced or even abolished when sodium is withdrawn from the bath solution and replaced by sucrose (Nastuk, 1953; del Castillo & Katz, 1955). Furukawa, Takagi & Sugihara (1956) showed that normal depolarization of the end-plate membrane could be produced by externally applied ACh in a sodium-free sucrose solution containing ammonium ion. ACh still reacted with the end-plate receptors and raised the ion permeability of the end-plate membrane, when sodium and chloride had been removed and the cell membrane completely depolarized by substitution of an isotonic potassium sulphate solution (del Castillo & Katz, 1955). These experimental results, as well as the present experiments, suggest that the external sodium ion is not indispensable in order to produce the end-plate responses to a motor nerve volley or to applied ACh. The role of the hydrazinium ion substituted for sodium ion, however, is obscure; it is probable that either the hydrazinium ion is acting in exactly the same way as sodium ion (as a charge carrier) or it increases the permeability of the membrane to calcium ion (or internal anions) during the end-plate potential.

Restoration of sodium-deficient frog nerve axons in hydrazinium solution was initially reported by Lorente de Nó *et al.* (1957). The restoration of neuromuscular transmission in hydrazinium solution shows that motor nerve endings can retain or regain excitability and release ACh in a sodium-free hydrazinium solution.

In view of the present experiments, neuromuscular transmission in the frog apparently can be maintained in the absence of sodium in the external media. It is, however, difficult to disprove the possibility that residual sodium may be retained in the intercellular space, particularly in the extracellular space of nerve fibres, even after prolonged soaking in sodium-free sucrose or hydrazinium solution. There is the further possibility that sodium ions may diffuse out from the interior of the muscle fibres, but it seems difficult to believe that an influx of residual sodium can be responsible for the excitability of the muscle fibre, including the post-synaptic membrane, and even for the excitability of the terminal nerve endings.

Neuromuscular transmission in hydrazinium solution was not maintained indefinitely; blockage occurred finally within 60–70 min. At this stage, however, the individual muscle fibres still maintained excitability and responded to direct intracellular stimulation and also to ACh applied in small concentrations. A slight depression of the sensitivity of the end-plate membrane to applied ACh, observed at this stage, does not seem to be the main reason for the blockage of transmission. It is probably caused mainly by inexcitability of the presynaptic elements. The blockage occurred even when no nerve stimulations were applied, suggesting that it is not caused by shortage of ACh in the motor terminals. That the amplitude of end-plate potentials was not decreased gradually but was abolished abruptly when repetitive nerve stimulations were applied may be accounted for by blockage of nerve conduction. Presumably conduction by the intramuscular nerve fibres was blocked near the terminal endings. Indeed, the threshold of nerve fibres, as in the muscle fibres, would be increased and conduction blockage could occur at the pre-terminal bifurcation by prolonged immersion in hydrazinium solution.

SUMMARY

1. When neuromuscular transmission in the frog's sartorius has been blocked by 20–180 minutes soaking in sodium-free isotonic sucrose, hydrazinium ions cause transmission to be restored within 5–10 minutes, though ultimately block occurs after about 1 hour in the hydrazinium solution.

2. Intracellular potential changes of muscle fibres induced by nerve and intracellular direct stimulation in hydrazinium solution closely resemble those in Ringer's solution, except that the former shows a slightly longer duration and smaller amplitude. The excitability of muscle fibres was well maintained after complete blockage of transmission occurred.

3. No appreciable changes of the resting potential and electrical properties of the muscle membrane were observed when it was immersed in hydrazinium solution for 180 minutes.

4. The end-plate potential in hydrazinium solution closely resembles that in Ringer's, except for slightly slower rising and falling phases.

5. No appreciable changes in the resting potential of the end-plate membrane were observed during 180 minutes soaking in hydrazinium solution.

6. The sensitivity of the end-plate membrane to applied ACh was well maintained in hydrazinium solution. There was, however, a slight tendency of the sensitivity to fall gradually during prolonged immersion (60–180 minutes).

7. Calcium is essential for the maintenance of neuromuscular transmission in sodium-free solution.

8. Blockage of transmission in hydrazinium solution did not seem to be caused by inexcitability of the post- or presynaptic membranes, but probably by conduction blockage which occurred in the terminal nerve axons.

9. It is suggested that the excitability of different kinds of excitable membranes can be maintained in media whose sodium ions are replaced by onium ions possessing a specific affinity to that membrane.

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THE EFFECT OF WIND SPEED ON MAXIMUM EVAPORATIVE CAPACITY IN MAN

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The rate of convective heat exchange between a man's skin and the surrounding air may be expressed as

$$H_c = k_c\,(T_s - T_a)$$

where H_c is the rate of heat transfer per unit area of skin,

T_s is the mean skin temperature

T_a the temperature of the air, and

k_c a coefficient depending on the air movement, the posture of the subject and his orientation to the wind.

Exchange of heat by evaporation of sweat from the skin follows similar laws provided that the skin is completely wet with sweat, as happens when the rate of sweat production considerably exceeds the rate of evaporation. If the skin is not completely wet the rate of evaporation will be less, so that the fully wet condition corresponds to the maximum rate of evaporation. Under conditions of incomplete wetness, the concept of maximum evaporative capacity remains useful, since the degree of skin wetness may be expressed as the ratio of the observed rate of evaporation to the maximum possible. For completely wet skin, then,

$$H_e = k_e(E_s - E_a),$$

where H_e is the rate of heat transfer by evaporation,

E_s is the vapour pressure at the skin surface (approximately saturation at T_s),

E_a is the water vapour pressure in the ambient air, and

k_e a coefficient analogous to k_c and depending on the same factors.

Where the areas available for heat exchange by convection and by evaporation are equal, it is to be expected that k_c and k_e will bear a constant ratio to one another, since both may be regarded as depending in part on the thickness of a hypothetical boundary layer of still air at the skin surface. This relationship will break down if the gradients of temperature or vapour pressure become very large (Jakob, 1949*a*), but should hold over the ranges encountered in human physiological investigations. The relation between k_c and k_e has been examined experimentally in man (Brebner, Kerslake & Waddell, 1958), and the results confirmed the statement by Woodcock, Powers & Breckenridge (1956) that k_e is twice k_c, when mm Hg and ° C are used respectively to define the gradients.

Previous determinations of the relation between wind speed and k_e and k_c have given results inconsistent with this. Nelson, Eichna, Horvath, Shelley & Hatch (1947) measured both coefficients at a series of different wind speeds. The relation between wind speed (V) and k_c was described equally well by the relations, $k_c = 0{\cdot}23V^{0{\cdot}62}$ and $k_c = 0{\cdot}53V^{0{\cdot}5}$. The observed values of k_e were described best by the relation $k_e = 1{\cdot}44V^{0{\cdot}37}$ and could not be fitted to equations containing $V^{0{\cdot}5}$ or $V^{0{\cdot}62}$. If k_c and k_e depend on different powers of wind speed, they will not remain in the same ratio to one another at different wind speeds. The authors were dissatisfied with their results on evaporation, and considered that they had probably failed to produce complete wetness at the higher wind speeds.

It was thought desirable to repeat the determination of k_e for standing subjects, using more severe heat stresses, which, it was hoped, would ensure complete wetting of the skin. The results are described in the present paper.

METHODS

Experiments were conducted in a booth situated in a wind tunnel (Brebner *et al.* 1958). This contained a recording balance, on the pan of which was a dish of liquid paraffin. The subject stood on the soles of a pair of plimsolls on a metal grid just above the fluid level, and all dripping sweat fell into the pan.

Temperature and humidity. Conditions were arranged to be severe enough to ensure that the skin was completely wet with sweat. At the same time it was necessary to preserve as large a vapour pressure gradient as possible between the skin and the air, in the interest of accuracy. A temperature of about 50° C was used throughout, and a humidity appropriate to each wind speed was found empirically. Air temperature and humidity were measured with an aspirating psychrometer upwind of the subject before and after each observation of weight loss.

Wind speed. The wind tunnel provided a linear horizontal air flow which was free from obvious turbulence. The air passed through a screen of wire gauze about 6 ft. (1·83 m) upstream of the subject. Wind speed was measured at 24 points distributed over a rectangle 2 ft. wide by 6 ft. high, situated 3 ft. upstream of the subject. A vane anemometer was used, which was subsequently calibrated against another newly calibrated by the N.P.L. The wind speed at all points was within 5% of the mean figure, and was not significantly affected by the presence or absence of the subject downstream. With the subject absent, the measurements were also made at the site normally occupied by him, and were found to agree with those made at the upstream position.

Skin temperature. Mean skin temperature was measured by means of a harness of 15 thermocouples having leads of equal length and connected in parallel to the same reference junction. Each couple consisted of an end-to-end copper-constantan junction to which thin elastic cords were tied. The thermocouples were all sited on convex body surfaces, and could be held in position by tying the elastic round the body. Tension was sufficient just to hold the couples in place, and deformation of the skin was slight. The sites of the thermocouples are shown in Fig. 1. The reference junction was held in a vacuum flask which stood beside the subject on the balance, and thin copper leads were run to a galvanometer. The weight and resilience of the leads were small, and since the balance was maintained in oscillation by its mechanism, their presence did not interfere with the recording of weight. The assembly was calibrated after each experiment.

Subjects. Three healthy young men were used, none of whom was very hirsute. Their heights, weights and surface areas are presented in Table 1.

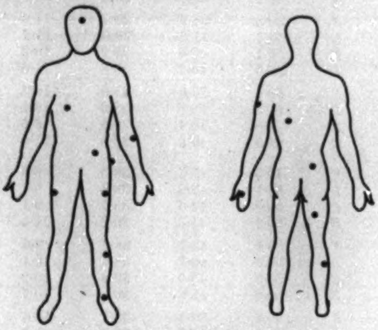

Fig. 1. Location of skin thermocouples.

Table 1. Characteristics of the subjects

Subject	Height (cm)	Weight (kg)	Surface area (Dubois) (m^2)
J.C.	170	68	1·79
J.W.	183	63	1·82
M.K.	177	79	1·97

Procedure. About 10 min after entering the booth, when inspection showed the skin to be wet with sweat, the subject took his place on the balance, standing in one of four orientations to the wind; front, back, right or left side into the wind. He stood with his feet a few inches apart and his arms hanging loosely, not touching his sides. After a further 5 min the balance was started, and skin temperature was recorded at 1 min intervals until an adequate weight record had been obtained. This took about 10 min. The change in skin temperature over this period was small, so that the assumption of a constant vapour pressure at the skin surface does not introduce significant error. The only quantities involved in the calculation of k_e are the vapour pressure gradient from skin to air and the rate of evaporation from the skin. No consideration of heat balance of the subject is involved, and no advantage would be gained by prolonging the exposure.

RESULTS

One determination of k_e was made on each subject in each orientation at each of four wind speeds. The order of presentation of orientation was selected at random, but all determinations at any one wind speed were made in sequence. The results are presented in Table 2. The effect of wind speed on the evaporation coefficient is large, but, at the same wind speed, the differences between subjects and between different orientations are not obvious. These effects are

TABLE 2. Experimental results

V	Subject	Orientation	T_a (° C)	E_a (mm Hg)	T_s (° C)	(E_s-E_a) (mm Hg)	H_e (kcal/m² hr)	k_e (kcal/m² hr mm Hg)
115 ft./min 35 m/min	J.C.	*F*	50·5	39·8	38·1	10·2	102	10·0
		L	50·3	39·3	38·8	12·7	113	9·0
		R	50·5	39·8	38·0	9·9	95	9·6
		B	50·1	41·0	39·1	11·7	104	8·9
	J.W.	*F*	50·8	38·9	38·3	11·6	110	9·5
		L	50·2	41·2	39·0	11·2	99	8·8
		R	50·1	40·0	38·2	10·2	90	8·8
		B	50·3	40·3	38·8	11·6	86	7·4
	M.K.	*F*	50·1	39·0	38·0	10·7	96	9·0
		L	50·1	39·5	38·0	10·2	97	9·5
		R	50·5	40·4	38·1	9·6	108	11·2
		B	50·3	40·5	38·8	11·4	91	8·0
208 ft./min 63 m/min	J.C.	*F*	49·5	42·5	38·5	8·5	114	13·4
		L	50·1	42·8	38·8	9·1	116	12·7
		R	50·0	42·5	38·7	9·1	118	13·0
		B	50·1	41·6	39·2	11·4	127	11·1
	J.W.	*F*	50·1	42·0	38·7	9·6	103	10·7
		L	50·0	40·7	38·4	10·1	135	13·3
		R	50·3	43·4	39·4	10·2	108	10·6
		B	50·0	42·9	39·4	10·7	119	11·1
	M.K.	*F*	50·2	39·4	38·1	10·6	130	12·3
		L	49·6	39·7	37·9	9·7	130	13·4
		R	49·0	40·5	37·8	8·7	166	19·1
		B	50·2	42·5	39·0	9·9	120	12·1
404 ft./min 123 m/min	J.C.	*F*	50·8	41·8	38·7	9·8	184	18·8
		L	51·0	43·0	38·5	8·0	163	20·4
		R	51·0	43·0	38·3	7·5	169	22·5
		B	50·9	43·3	38·5	7·7	163	21·2
	J.W.	*F*	50·9	43·0	38·3	7·5	166	22·1
		L	50·8	43·0	38·1	7·0	153	21·9
		R	49·3	43·9	38·7	7·7	146	19·0
		B	51·0	44·0	38·7	7·6	163	21·4
	M.K.	*F*	50·7	43·0	38·1	7·0	162	23·1
		L	50·7	43·0	38·3	7·5	190	25·3
		R	51·0	43·7	38·4	7·1	161	22·7
		B	50·4	43·0	38·8	8·9	176	19·8
790 ft./min 241 m/min	J.C.	*F*	50·7	47·4	39·6	6·8	181	26·6
		L	50·4	47·6	39·4	6·0	174	29·0
		R	50·5	47·4	39·3	5·9	188	31·9
		B	49·4	46·5	39·0	5·9	157	26·6
	J.W.	*F*	50·2	48·5	39·6	5·7	181	31·8
		L	49·0	46·2	39·1	6·5	173	26·6
		R	49·1	48·0	39·2	5·0	150	30·0
		B	49·1	46·8	39·0	5·6	176	31·4
	M.K.	*F*	50·7	47·0	39·4	6·6	194	29·4
		L	50·0	45·7	38·9	6·5	212	32·6
		R	50·4	47·2	39·2	5·8	179	30·9
		B	49·3	46·0	38·7	5·6	173	30·9

V, wind speed; orientation, F front, L left side, R right side, B back into wind; T_a, air temperature; E_a ambient humidity; T_s, mean skin temperature; E_s-E_a, vapour pressure gradient from wet skin to ambient humidity; H_e, heat loss by evaporation, k_e, coefficient for heat exchange by evaporation.

summarized in Table 3, which shows mean values of k_e, at each wind speed for each orientation (mean of three subjects) and for each subject (mean of four orientations). The largest subject, M.K., has the largest value of k_e at all wind speeds, but the difference between him and the others is not very great. There is no consistent pattern in the results for different orientations and the differences are comparable with the experimental error.

TABLE 3. Variation of k_e with orientations and with subjects. k_e is the coefficient for heat exchange by evaporation (kcal/m² hr mm Hg)

	Mean values of k_e at four wind speeds			
ft./min	115	208	404	790
m/min	35	63	123	241
Orientation				
F	9·5	12·1	21·3	29·3
L	8·9	13·1	22·5	29·4
R	9·9	14·2	21·4	30·9
B	8·1	11·4	20·8	29·6
Subject				
J.C.	9·4	12·5	20·7	28·5
J.W.	8·6	11·4	21·1	29·9
M.K.	9·4	14·2	22·7	30·9

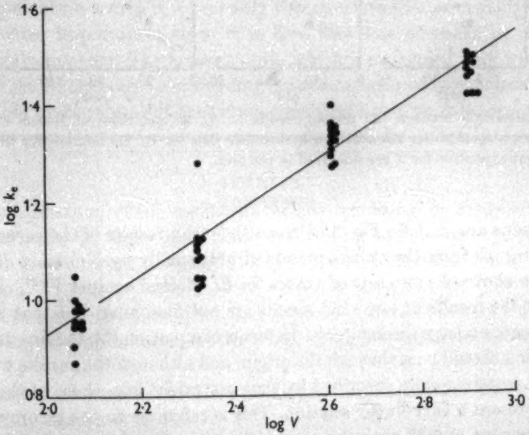

Fig. 2. The relation between log k_e and log V. k_e is the coefficient for heat exchange by evaporation; V is wind speed. Observations on all subjects at all orientations are shown. Some points have been displaced to the right to avoid superimposition. The regression line is described by log k_e = 0·634 log V − 0·3509.

For the practical purpose of expressing a relation between k_e and V, results for different subjects and orientations may be considered together. It is to be expected (Nelson *et al.* 1957) that the relation will be of the form

$$k_e = aV^b. \qquad (1)$$

The constants a and b may be determined by plotting log k_e against log V. The resulting line should be straight, with a slope b and intercept log a. The results are presented in this form in Fig. 2. The regression line provides values for insertion into equation (1)

$$k_e = 0{\cdot}446\,V^{0{\cdot}634 \pm 0{\cdot}024}. \tag{2}$$

If this is a true description of the findings, a plot of k_e against $V^{0{\cdot}634}$ should produce a straight line, with a slope of 0·446 and this should pass through the origin. It will be noticed that if this line has a significant intercept, the log/log plot is invalidated, since it should not in this case, yield a straight line. The intercept is in fact insignificant, and the slope is 0·435 ± 0·056, consistent with equation (2). The best line through the origin has a slope of 0·446.

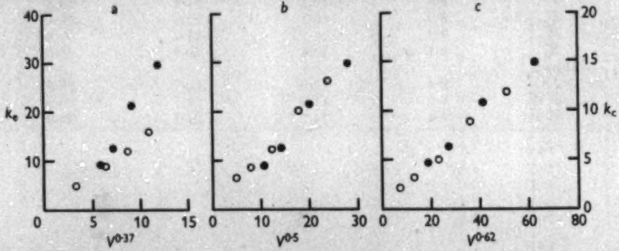

Fig. 3. Comparison between the present results for k_e, ●, and those of Nelson *et al.* (1947) ○, for (*a*) k_e, (*b*, *c*) k_c. The scale for k_c is double that for k_e. The reasons for the choice of different exponents for V are described in the text.

DISCUSSION

Comparisons are made in Fig. 3 between the mean results of the present series and the means from the values presented graphically by Nelson *et al.* (1947). Figure 3*a* shows the two sets of values for k_e, plotted against $V^{0{\cdot}37}$. It will be seen that the results at low wind speeds are not dissimilar, but that at higher speeds there is a large discrepancy. In terms of equation (1), the line describing the relation should pass through the origin, and although the results of Nelson *et al.* can be adequately described by such a straight line, those of the present series represent a curvilinear relation. This is referable to the incompatibility of an exponent of 0·37 with the results shown in Fig. 2.

Figure 3*b* and *c* shows mean values for k_e from the present series and those for k_c from Nelson *et al.* (1947). The latter are plotted on a scale twice as large as that for k_e, since it is to be expected that k_e shall be twice k_c at all values of V (Woodcock *et al.* 1956). Agreement is good using $V^{0{\cdot}62}$ (Fig. 3*c*), and is not too bad using $V^{0{\cdot}5}$ (Fig. 3*b*), although there is a significant difference between 0·5 and 0·634 ± 0·024, the exponent derived for the present series from Fig. 2.

There is thus agreement between the values of k_c at wind speeds between

25 and 600 ft./min determined by Nelson *et al.* (1947) and the present determination of k_e for wind speeds between 100 and 800 ft./min (30·5, 244 m/min). The two coefficients bear the expected relation to one another, and it is reasonable to suppose that both determinations are satisfactory, i.e. that the skin was completely wet in the present series, and that neither series contains important systematic errors. The direct determination of k_e by Nelson *et al.* (1947) was recognized by them to be unsatisfactory, since the skin was thought to be incompletely wet at the higher wind speeds. It appears from Fig. 3*a* that this was so, and also that full wetness probably was achieved at the lower wind speeds.

While the conditions used in the present work were more severe than would be necessary in order just to achieve full wetness, it seems probable that at moderate and high wind speeds any environment which produced complete wetting of the skin would be so severe as to be tolerable only for short periods. The subjects of the present series were frequently near to collapse at the end of their 20–25 min exposure. The upstream and downstream surfaces of a cylinder standing in a wind have been found to have larger values of k_c than the sides (Jakob, 1949*b*), and for the assessment of the over-all heat stress of an environment on a man it is probably the poorly ventilated regions that are of the greater importance, since it is here that loss of sweat by dripping is more likely to occur. The present work, which is concerned only with mean values for the whole body, is misleading in the simplicity of its conclusions, and it is possible that systems of heat stress appraisal based on mean evaporation coefficients may lead to erroneous conclusions at moderate levels of stress.

SUMMARY

1. Determinations of the coefficient for heat exchange by evaporation, k_e, have been made on three nude subjects standing at four orientations to a horizontal wind at wind speeds of 115, 208, 404 and 790 ft./min (35, 63, 123, 241 m/min).

2. Differences between subjects and between orientations were small.

3. Taking all results together, the effect of wind speed is described by the equation $k_e = 0{\cdot}45V^{0{\cdot}63}$.

4. Results are consistent with the previous determinations of the convection coefficient by Nelson *et al.* (1947).

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STUDIES ON IODIDE TRANSPORT AGAINST A CONCENTRATION GRADIENT BY THE SMALL INTESTINE OF THE RAT *IN VITRO*

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Many vertebrate tissues are known to transport iodide against a concentration gradient, notably thyroid (Vanderlaan & Vanderlaan, 1947), stomach (Davenport, 1943), salivary glands (Fletcher, Honour & Rowlands, 1956), mammary glands (Honour, Myant & Rowlands, 1952) and placenta (Logothetopoulos & Scott, 1956). Our experiments were designed to test whether any region of the small intestine was capable of carrying out the same process *in vitro*. It was expected that such a study would enable the mechanism of iodide transport to be investigated in conditions which could be controlled relatively easily. While this work was in progress, Pastan (1957) reported that carrier-free radio-iodide passes *in vivo* against a concentration gradient into the lumen of the central region of the rat's small intestine after parenteral injection. Our observations have shown that this process occurs *in vitro*. We have also studied the changes in the rate of iodide transport which result from altering the concentration of carrier iodide. Finally, experiments are presented which throw some light on the relation between iodide transport against a concentration gradient and other metabolic processes.

METHODS

From the small intestine of each of a series of albino rats, killed by a blow on the head, ten everted sacs of approximately equal size (numbered from the duodenal end) were prepared according to the procedure of Wilson & Wiseman (1954). The Krebs-Ringer bicarbonate solution contained $Na^{131}I$ 1 μc/ml. (approx.) and, unless otherwise stated, glucose 0·3 % (w/v). It was gassed with O_2 95 + CO_2 5 %. In some experiments no carrier iodide was present. In others the medium contained iodide (0·001–10 mM) added as the sodium or potassium salt. The sacs held approximately 1 ml. of Krebs-Ringer solution (the 'serosal fluid') in equilibrium with the serosa, which is on the inside of the everted intestinal sac. The filled sacs were placed in Warburg cups containing 3 ml. of Krebs-Ringer solution (the 'mucosal fluid'), identical, unless otherwise stated, with that used to fill the sacs. After 1 hr at 37° C (shaking speed 180/min) the sacs were taken from the Warburg cups and opened, and the serosal fluid was weighed. Pilot experiments showed that, on the average, 96% of the fluid injected into a sac was recovered in this way. The final volume of the serosal fluid was corrected to compensate for this loss. The amounts of radioactivity present in the original Krebs–Ringer solution and in the serosal and mucosal fluids at the end of the experiment were measured in a liquid counter (Veall, 1948) after 1:20 or 1:50 dilution. The terminal portions of intestine beyond the ligatures were discarded and the sacs were dried and weighed. Radioactivity in the dried sacs was measured after digestion at 90° C in potassium hydroxide 5% (w/v). From these observations the changes in the iodine content of the mucosal and serosal fluids and the amount of iodine taken up by the gut wall were calculated. Simply for convenience, these changes were expressed as μ-equiv of iodide $\times 10^{-3}$/mg dry wt./hr. It should be realized, however, that the radioactivity measurements by themselves did not allow any conclusions to be drawn about the chemical state of the radio-iodine at the end of an experiment.

Some systematic errors were involved in estimating the changes in the iodine content of the mucosal fluid. First of all, the water taken up by the intestinal wall could not be estimated since the sacs were not weighed before and after the experiment because of the added risk of radioactive contamination in the laboratory which this procedure would entail. As a result, the final mucosal fluid volume, and hence its total iodine content, were overestimated. Secondly, the terminal sections of the intestine beyond the ligature take up iodine, thereby causing a loss of iodine from the mucosal fluid. Hence, transport rates, where given, were calculated by subtracting the amount of iodine taken up by the sac wall from the total amount lost by the serosal fluid.

In duplicate experiments, where the original $K^{127}I$ concentration was 1 μM and the $Na^{131}I$ concentration was about 0·8 μc/ml., the chemical nature of the transported iodine was investigated chromatographically. One-dimensional chromatograms were prepared on Whatman No. 3 MM paper from 10 μl. portions of the mucosal fluids corresponding to each sac, using the descending method. The developing solvent system was *n*-butanol:ethanol:2N-NH_4OH (10:2:4 by vol.) at 32° C. Radioautograms were then prepared by exposing the chromatograms on 'Ilfex' double-coated X-ray films for 14 days.

RESULTS

In all the quantitative experiments reported below the iodine content of any solution was estimated by measuring its radioactivity. It was therefore necessary to show that none of the iodide present at the beginning of the experiment had undergone a chemical change which could simulate iodide transport against a concentration gradient.

The chromatographic experiment showed that the areas of the iodide spots in the mucosal fluids corresponding to segments 3–6 were appreciably increased in comparison with the blank, indicating that more radioactivity was present in the former case. In these fluids only one single spot was present whose R_F was 0·5. Hence, the increase in radioactivity of these mucosal fluids almost certainly resulted entirely from an increase in their iodide content. In the original Krebs-Ringer solution and, more obviously, in segments 7–10, a faint additional spot was present ($R_F \simeq 0{\cdot}6$), whose nature is at the moment uncertain. In this particular experiment liquid counting after 1:20 dilution of selected mucosal fluids gave the following results: segment 3, 241·2 counts/sec; segment 4, 226·6 counts/sec; segment 5, 206·9 counts/sec; segment 6, 211·5 counts/sec; segment 10, 158·8 counts/sec.

Table 1 gives the results, treated statistically, of 11 one-hour experiments, showing the changes in iodide content when the serosal and mucosal fluids were initially identical in composition. The general trend is obvious, although there is considerable variation between different runs. The central parts of the small intestine transport iodide against a concentration gradient from the serosal to the mucosal side, the transport rate being maximal in the jejunal region. In individual experiments iodide transport against a concentration gradient was usually observed in sacs 3–6.

Table 1. Iodide transport by everted sacs of rat small intestine. Sacs were incubated for 1 hr at 37° C in NaI 0·1 mM, labelled with ^{131}I, dissolved in Krebs-Ringer bicarbonate solution gassed with O_2 95% + CO_2 5% and containing glucose 0·3% (w/v). The results of 11 experiments are given. Intestinal segments were numbered from the duodenal end

	Changes in I′ content (μ-equiv $\times 10^{-3}$/mg dry wt./hr)						H_2O transport (μl./mg dry wt./hr)	$\frac{I'_{mucosal}}{I'_{serosal}}$
	Serosal fluid		Mucosal fluid		Intestinal wall			
Segment	Mean	s.e. of mean	Mean	s.e. of mean	Mean	s.e. of mean	Mean	Mean
1	−0·05	0·06	−0·34	0·07	0·27	0·02	2·46	1·34
2	−0·21	0·06	−0·16	0·07	0·26	0·02	2·73	1·81
3	−0·6	0·11	0·12	0·05	0·20	0·02	2·85	2·70
4	−0·81	0·09	0·44	0·06	0·14	0·02	2·40	4·08
5	−0·94	0·10	0·53	0·10	0·10	0·01	2·36	4·90
6	−0·81	0·11	0·28	0·06	0·12	0·01	2·72	4·11
7	−0·55	0·04	0·15	0·05	0·14	0·01	2·03	2·67
8	−0·16	0·06	−0·29	0·17	0·24	0·01	1·94	1·59
9	−0·12	0·06	−0·42	0·11	0·24	0·02	2·08	1·21
10	−0·12	0·15	−0·29	0·08	0·20	0·03	0·97	1·15

Table 2. Iodide transport by everted sacs of rat small intestine. Krebs–Ringer bicarbonate solution containing glucose 0·3% (w/v) was used as serosal and mucosal fluids. Only the mucosal fluid contained ^{131}I-labelled NaI (0·1 mM), hence the transport of iodide was in this case from the mucosal to the serosal side. Sacs were numbered from the duodenal end

	I′ transport rate (μ-equiv $\times 10^{-3}$/mg dry wt./hr)			
	Expt.			
Sac no.	*A*	*B*	*C*	Mean
1	0·31	0·35	0·30	0·32
2	0·23	0·27	0·21	0·24
3	0·06	0·11	0·08	0·08
4	0·06	0·05	0·05	0·04
5	0·03	0·04	0·03	0·03
6	0·04	0·03	0·05	0·04
7	0·08	0·09	0·20	0·12
8	—	0·05	0·33	0·19
9	0·55	0·41	—	0·48
10	0·28	0·04	0·11	0·14

Table 2 shows that, when iodide is initially present on the mucosal side only, passage of iodide from the mucosal to the serosal side does occur, even in those regions of the intestine which can transport iodide most rapidly against a concentration gradient from the serosal to the mucosal side. In these conditions, as might be expected, the transport rate is slower in the central parts of the intestine.

Relation between the initial iodide concentration and the iodide transport rate

Table 3 shows that as the initial concentration of iodide rises, the iodide transport rate increases up to an apparent maximum when the iodide concentration is 1 mM. Only measurements on the sac in each experiment with the highest concentration ratio were used to construct the table. Because of differences in serosal fluid volume, these sacs were not necessarily the ones showing the highest transport rates.

TABLE 3. Effect of increasing iodide concentration on iodide transport by everted sacs of rat small intestine

Iodide transport rates are estimated by deducting the amount of iodide taken up by the intestinal wall from the iodide lost by the serosal fluid. The figures refer only to the sac in each experiment with the highest iodide concentration ratio (not transport rate).

I′ concn. (mM)	No. of expts.	Concn. ratio $\frac{I'_{mucosal}}{I'_{serosal}}$		I′ transport rate (μ-equiv $\times 10^{-3}$/mg dry wt./hr)		% of total serosal I′ transported (mean)
		Mean	Range	Mean	Range	
Carrier-free	3	10·4	8·5–13·2	—	—	75
0·001	3	10·0	8·0–13·9	0·011	0·008–0·015	80
0·01	3	11·1	7·3–13·9	0·081	0·076–0·089	65
0·05	3	13·7	12·3–14·9	0·34	0·07 –0·77	77
0·1	11	5·4	3·2– 7·6	0·77	0·38 –1·41	57
1	3	4·7	2·0– 7·6	4·2	3·5 –4·5	40
10	3	1·9	1·7– 2·0	3·3	1·2 –4·9	3

Relation between iodide transport and intestinal metabolism

Table 4 shows that the iodide transport rate remains high when glucose is omitted from the Krebs-Ringer solution but is greatly reduced in the presence of sodium cyanide (2 mM). It is, however, negligible in anaerobic conditions and in the presence of potassium perchlorate (0·1 mM). The last-named substance was observed by Pastan (1957) to inhibit active iodide transport *in vivo*. Iodide transport against a concentration gradient still occurred in the presence of methylthiouracil (1·3 mM).

DISCUSSION

The results of Table 1 show that iodide transport against a concentration gradient can be carried out by the central region of the small intestine of the rat *in vitro*. It is reasonable to conclude from the chromatographic experiments that the transported ^{131}I appears in the mucosal fluid as iodide and not in any other form. 'Unknown 2' (Taurog, Tong & Chaikoff, 1957), which is separated from iodide by the *n*-butanol:ethanol:ammonia solvent system, was not detected in the central region of the small intestine, where the concentration process occurred. The small additional spot ($R_F \simeq 0{\cdot}6$) present in the original fluid and in the mucosal fluid of the inactive segments 7–10 might possibly be 'Unknown 2', though any firm identification must await the chemical characterization of both substances.

Logothetopoulos & Myant (1956), in contrast to Pastan (1957), did not observe iodide transport against a concentration gradient into the lumen of the rat's small intestine. However, the former workers, unlike Pastan (1957), did not take the precaution of dividing the small intestine into sections by means of ligatures. Hence it is likely that the passage of the intestinal contents along the lumen of the intestine would have obscured the effects of the localized iodide concentration mechanism. This probably explains why Logothetopoulos & Myant failed to detect transport of iodide against a concentration gradient in the intestine. It is of interest that, in a study of glucose absorption, Reynell & Spray (1956) assumed without further investigation that only simple diffusion processes are involved in iodide absorption and used results on iodide to provide control values for comparison with active glucose absorption.

TABLE 4. Relation between iodide transport and oxidative metabolism in everted sacs of rat small intestine

In all cases the Krebs-Ringer bicarbonate solution contained NaI 0·1 mM. Other experimental conditions are given in the first column. Individual entries in cols. 3–5 are means of the number of experiments tabulated in col. 2. Only observations on the sac with the highest concentration ratio in each experiment were used to construct the table. Changes in the iodide content of the serosal and mucosal fluids and of the intestinal wall are recorded. Water transport was measured as the change in volume of the serosal fluid.

1	2	3	4			5
		Concn. ratio	Changes in I′ content (μ-equiv × 10^{-3}/mg dry wt./hr)			H_2O transport
Experimental conditions	No. of expts.	$\frac{I'_{\text{mucosal}}}{I'_{\text{serosal}}}$	Mucosa fluid	Serosal fluid	Intestinal wall	(μl./mg dry wt./hr)
Aerobic (O_2 95 + CO_2 5%) Glucose (0·3% w/v)	11	5·4	+0·51	−0·88	+0·11	+2·4
Aerobic (O_2 95 + CO_2 5%) No glucose	3	4·0	+1·06	−1·16	+0·11	+1·6
Anaerobic (N_2 95 + CO_2 5%) Glucose (0·3% w/v)	3	1·1	−0·04	−0·35	+0·29	−1·4
Aerobic (O_2 95 + CO_2 5%) Glucose (0·3% w/v) NaCN (2 mM)	3	1·4	+0·52	−0·64	+0·34	−0·8
Aerobic (O_2 95 + CO_2 5%) Glucose (0·3% w/v) $KClO_4$ (0·1 mM)	3	1·7	−0·16	−0·24	+0·15	+4·0
Aerobic (O_2 95 + CO_2 5%) Glucose (0·3% w/v) Methylthiouracil (1·3 mM)	3	4·5	+0·26	−0·66	+0·10	+2·89

Table 1 shows that iodide uptake *in vitro* by the intestinal wall is lowest in the central region, where iodide transport is most rapid. Pastan (1957), on the other hand, observed maximum iodide uptake *in vivo* to occur in the central region of the intestinal wall. These contradictory findings can be reconciled in the following way. More iodide is probably taken up by the intestinal wall from the serosal fluid than from the mucosal fluid, since the former would be expected to be in equilibrium with a greater volume of tissue. Consequently, *in vitro*, differences between the amounts of iodide present in the mucosa of different parts of the intestine would be obscured by the variations in serosal iodide concentration. *In vivo*, however, the serosal iodide concentration would be expected to follow the blood iodide concentration and therefore would remain constant along the whole length of the intestine, in which conditions the differences between the iodide content of the mucosa in different regions would be detected.

Table 2 confirms the finding of Pastan (1957) that iodide absorption can take place from all parts of the rat's small intestine, although at a much slower rate in the central region.

Roche & Michel (1955) consider iodide itself to be an inhibitor of iodide-concentration mechanisms, over and above its effect on the synthesis of organically bound iodine. This concept was probably based on measurements of the concentration ratio only and the results of Table 3 show that it may not be entirely correct. It would seem that the existence of a maximum limit to the active iodide transport rate would explain *in vivo* observations, such as those of Vanderlaan & Vanderlaan (1947), showing a progressive reduction in the iodide concentration ratio of the thyroid gland as the plasma iodide concentration increases beyond a certain point. Such results would be expected once the active iodide transport rate had reached its maximum, provided that there is no barrier to the simple diffusion of iodide between plasma and thyroid.

The maximum concentration ratio attainable by everted sacs of rat small intestine appears to be about 14. This is low in comparison with the values of 25 and 250, the respective iodide concentration ratios observed in the thyroid glands of normal and propylthiouracil-treated rats (Vanderlaan & Vanderlaan, 1947). The iodide concentration ratio in the salivary glands *in vitro* is 5–10 (Fletcher *et al.* 1956), though for saliva *in vivo* it was found usually to lie within the range 10–60, although a few higher values (up to about 100) were observed (Honour *et al.* 1952). The iodide concentration ratios for gastric juice and milk both reach a maximum of about 30 (Honour *et al.* 1952), but the placenta concentrates iodide only some four to five times (Logothetopoulos & Scott, 1956). In view of these wide variations in iodide concentrating powers, it is possible that the iodide metabolism of different tissues may differ qualitatively as well as quantitatively, although no evidence for or against this supposition is at present available.

It should be noted that all the above-mentioned work on iodide-concentrating processes was carried out before the presence of 'Unknown 2' (Taurog, *et al.* 1957) was suspected. This unknown substance has similar chromatographic properties to iodide in most of the usual solvent systems. It appears to be an oxidation product of iodide and is synthesized by homogenates and particulate suspensions prepared from many organs. Hence in some cases the iodide concentration ratios which have been observed might not be true indices of iodide transport, if the underlying process were wholly or in part the synthesis of 'Unknown 2' and not the transport of iodide against a concentration gradient. This does not apply to the present investigation in which the presence of 'Unknown 2' has been excluded in the active regions of the small intestine.

If a carrier substance is involved in iodide transport, it may not necessarily be specific to iodide. Chloride is known to pass isosmotically in the opposite direction from iodide, i.e. from mucosal to serosal side (Wilson, 1954). If this process were carrier-mediated, it would imply that the concentration of 'free' carrier (not in combination with chloride) increased on passing from the mucosal to the serosal side of the intestine. This free carrier might then transport iodide with it as it diffused back to the mucosal side of the cell, provided that iodide could combine with the carrier molecule in the same way as chloride. In general terms, iodide must undergo some chemical transformation at the serosal side of the cell and then diffuse across to the mucosal side, where it is released into the mucosal fluid. The unidentified compound 'Unknown 2' (Taurog *et al.* 1957) might possibly be concerned in this process.

The results in Table 2 make it seem unlikely that a barrier impermeable to iodide exists in any part of the mucosal cell. Hence there must be an equilibrium between simple diffusion of iodide on the one hand and an active process on the other, the latter being responsible for the passage of iodide against a concentration gradient in one direction only, i.e. from the serosal to the mucosal side.

Iodide transport against a concentration gradient is an aerobic process and in large part appears to be cyanide-sensitive. It is apparently unaffected by changes in the rate of water transport or by the absence of glucose from the fluid medium, in which latter case little or no lactic acid passes from the mucosal cells into the serosal fluid. Hence iodide ions are probably not being exchanged for lactate ions. The synthesis of organic iodine compounds appears to play no part in iodide transport.

Potassium perchlorate in low concentrations inhibits the transport of iodide against a concentration gradient without having any obvious effect on the metabolism of intestinal sacs, as judged by water transport, which is usually a most sensitive index of viability. This inhibition might result from competitive combination of the perchlorate ion with an iodide carrier, protein in nature, since iodide has the lower position in the Hofmeister series. This hypothesis is, however, purely speculative. A similar inhibition of iodide transport by perchlorate without any apparent alteration in oxidative metabolism has, been reported in salivary glands (Fletcher *et al.* 1956).

There remain three problems concerning iodide transport against a concentration gradient by the small intestine of the rat. First its detailed mechanism must be elucidated. Secondly, its relation to the cyanide-sensitive respiratory processes of the intestinal cells must be clarified. And thirdly, some explanation must be found for the differences in rate of iodide transport shown by different regions of the rat's small intestine.

SUMMARY

1. Iodide transport against a concentration gradient by everted sacs of rat small intestine was studied *in vitro*.

2. Iodide transport against a concentration gradient from the serosal to the mucosal side was observed in the jejunum, usually in sacs Nos. 3–6 out of a total of ten intestinal sacs, numbered from the duodenal end.

3. In one-hour experiments, the concentration ratio $\frac{[I']_{\text{mucosal}}}{[I']_{\text{serosal}}}$ was found to reach a maximum value of about 14 when the initial iodide concentration was 50 μM or less.

4. The iodide transport rate was found to reach a maximum of about $4 \cdot 5 \times 10^{-3}$ μ-equiv/mg dry wt./hr when the initial iodide concentration was 1 mM.

5. Iodide transport against a concentration gradient was negligible in anaerobic conditions and greatly reduced in the presence of sodium cyanide 2 mM.

6. Potassium perchlorate 0·1 mM was found to inhibit iodide transport against a concentration gradient.

7. Iodide transport against a concentration gradient occurred in the absence of glucose from the fluid medium.

8. Iodide transport against a concentration gradient was unaffected by the presence of methylthiouracil 1·3 mM in the fluid medium.

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MUSCLE AND SKIN BLOOD FLOW IN THE HUMAN FOREARM DURING INSULIN HYPOGLYCAEMIA

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Studies with the venous occlusion plethysmograph have shown that insulin hypoglycaemia is associated with an increased blood flow to the forearm and calf, and less consistently, to the hand and foot (Abramson, Schkloven, Margolis & Mirsky, 1939; French & Kilpatrick, 1955; Allwood, Ginsburg & Paton, 1957). With this technique it was only possible to measure the total blood flow within the segment inside a plethysmograph. Allwood, Birchall & Staffurth (1958) used local ^{24}Na clearance in an attempt to determine whether the vasodilatation in the forearm occurred in the skin, or in muscle, or both, but this method did not allow circulatory changes to be followed in the skin and muscle of the same forearm concurrently. This has now been accomplished in the present investigation, in which changes in blood flow have been recorded simultaneously from forearm skin and muscle during insulin hypoglycaemia using heat-conductivity measurements.

METHODS

The subjects were sixteen healthy young adults of both sexes. They lay recumbent, wearing light attire and covered with a blanket, in a room maintained at approximately 23° C. They had not eaten for 3 hr; a few had fasted since the previous evening. All were given soluble insulin (Hoechst) 0·1 i.u./kg I.V., receiving on the average 8 i.u. Skin blood flow was recorded with a thermal conductivity meter (Hensel & Bender, 1956) from the middle third of the forearm. Muscle blood flow was followed with a calorimetric probe (Hensel & Ruef, 1954; Hensel & Bock, 1955) in the brachioradialis-extensor group of muscles. In the majority of experiments the pulse rate was continuously recorded with an electronic pulse-rate meter (Fr. Müller & Zaun, Marburg). Blood sugar was estimated at frequent intervals by the Hagedorn–Jensen method. The latter results are not discussed in detail.

At the start and finish of each experiment the circulation to the arm was occluded for 3 min and the resulting changes in thermal conductivity, including those during the hyperaemia following occlusion, used as reference points for evaluating changes in blood flow. The effects of adrenaline 10 μg I.V. given into the opposite arm were treated similarly.

RESULTS

With the exception of six subjects there appeared, approximately 30 min after the injection of insulin, the well known symptoms of hypoglycaemia, including anxiety, restlessness, hunger, sensations of heat and palpitations. Blood sugar decreased, reaching its lowest value at the same time. Physical signs included tachycardia, visible carotid pulsations, hyperpnoea, marked flushing of the face, and sweating. In the six exceptions mentioned above the dose of insulin administered did not reduce the blood sugar level to the 'critical point', and subjective and objective signs of hypoglycaemia were absent; these experiments have not been taken into consideration in working out the results.

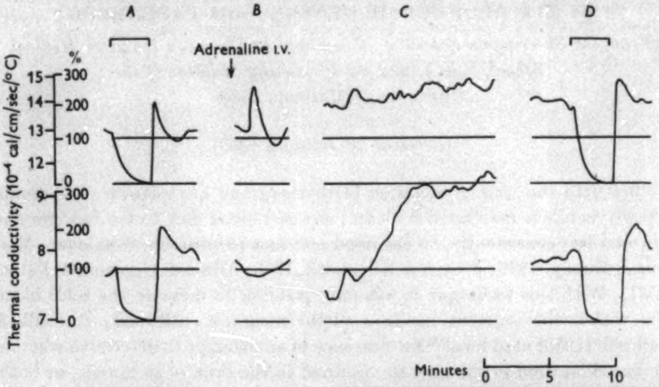

Fig. 1. Part of the record from an experiment showing changes in muscle blood flow (above) and cutaneous blood flow (below) following various procedures. *A*, response to 3 min arterial occlusion; *B*, response to adrenaline 10 µg I.V.; *C*, 25–35 min after insulin 8 i.u. I.V.; *D*, 50 min after insulin, and response to 3 min arterial occlusion.

After the injection of insulin the skin and muscle blood flow remained unchanged for the first 20 min. During the next 10 min blood flow in the skin decreased to 65% of the mean resting value, while muscle blood flow invariably increased, reaching a maximum about 30 min after the injection of insulin at an average of 175% (S.E. ± 5·97) of the resting level, a difference which is highly significant ($P < 0{\cdot}001$). At about this time, which coincided with the onset of more marked subjective sensations, cutaneous blood flow began to increase rapidly up to an average of 212% (S.E. ± 8·24) of the resting value, an increase which is also highly significant ($P < 0{\cdot}001$). The blood flow in the skin reached a peak 2–3 min after that in the muscle. During the next 10 min skin and muscle blood flow either remained at this high level, or decreased slightly. Part of the record from a typical experiment is shown in Fig. 1. Thereafter, as the blood sugar returned to normal, subjective sensations passed off and both the skin and muscle blood flows also returned to normal: initial values were attained about 60–70 min after the injection of insulin. The mean response at different times after the injection is illustrated in Fig. 2.

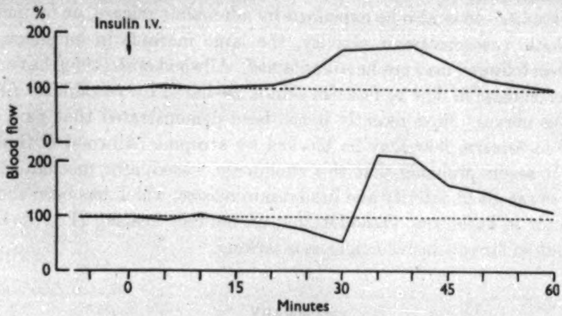

Fig. 2. The average change in muscle blood flow (above) and in cutaneous blood flow (below) following I.V. insulin 0·1 i.u./kg given at zero time.

DISCUSSION

These experiments show that the increase in forearm blood flow which occurs during insulin hypoglycaemia is located in blood vessels both of the skin and of muscle. An increase in cutaneous blood flow at this time has previously been demonstrated with the isotope-clearance technique by Allwood *et al.* (1958), but their results from forearm muscle were inconclusive, although an increased clearance from muscle generally occurred. Since an increased blood flow to muscle was invariably found in the present series during the hypoglycaemic reaction a comparison of the thermal-conductivity and isotope-clearance methods would be of interest.

Although the present experiments were not made with the object of determining the cause of the increase in peripheral blood flow during hypoglycaemia, the finding that vasodilatation occurs both in skin and muscle sheds light upon it. According to Allwood *et al.* (1957) both humoral and nervous factors are concerned in this increase. Of humoral factors adrenaline seemed the most likely, for there is direct evidence of adrenaline release in animals and in man during hypoglycaemia (von Euler & Luft, 1952; Holzbauer & Vogt, 1954). The finding that muscle blood flow increased in hypoglycaemia may be explained, in part, by adrenaline release. However, it is questionable whether it can be attributed solely to adrenaline, for the increase in muscle flow at the height of the reaction was often as great as that attained during the peak dilatation following the injection of adrenaline 10 μg I.V. Ginsburg & Paton (1956) have shown that forearm blood flow increased during hypoglycaemia in patients after bilateral adrenalectomy, but it is not known whether this vasodilatation was mediated by the sympathetic nerve supply to muscle or by some other humoral agent.

While the initial decrease in skin blood flow—which was not revealed by isotope studies—may also be explained by adrenaline release, or by increased sympathetic vasoconstrictor activity, the large increase in cutaneous flow which then followed may not be so explained. Allwood *et al.* (1958) have shown that this increase in flow to forearm skin is mediated by vasodilator fibres in cutaneous nerves. More recently it has been demonstrated that part of the increase in forearm flow may be blocked by atropine (Allwood & Ginsburg, 1959). It seems probable that this cholinergic vasodilator mechanism may involve sweat-gland activity and bradykinin release, which has been shown to be a factor in cutaneous vasodilatation on indirect heating (Fox & Hilton, 1958), but so far conclusive evidence is lacking.

SUMMARY

1. Blood flow was recorded simultaneously in skin and muscle of the human forearm during insulin hypoglycaemia, using thermal-conductivity meters.

2. Cutaneous blood flow, after an initial decrease of short duration, increased rapidly during hypoglycaemia to twice the resting value.

3. Muscle blood flow increased at the same time to 175% of the resting level. Comparison with the response to I.V. adrenaline suggests that the increased muscle flow at this time is not due solely to adrenaline release.

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RESUSCITATION OF DOGS FROM SEVERE ACUTE CARBON MONOXIDE POISONING

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The relative merits of pure oxygen and of a mixture of oxygen with carbon dioxide, in resuscitation from carbon monoxide poisoning, have been the subject of debate for thirty or forty years. Many papers have been published on the subject (Henderson & Haggard, 1920; Final Report of the Commission on Resuscitation from Carbon Monoxide Asphyxia, 1923; Sayers & Yant, 1923; Drinker & Shaughnessy, 1929; Sayers & Davenport, 1930; Campbell & Poulton, 1934) but some of those describing animal experiments have been somewhat unconvincing for various reasons.

Amongst the earlier authors Henderson & Haggard (1920) based their conclusions on experiments using only a small number of dogs; two dogs breathed oxygen, and two breathed 10% CO_2 in oxygen. The blood CO fell more rapidly in the dogs breathing the CO_2 mixture. Nicloux and his co-workers (Nicloux, Nerson, Stahl & Weill, 1925*a*, *b*) compared one dog breathing oxygen with one breathing 5% CO_2 in oxygen; they too, found a more rapid fall in blood CO in the dog breathing the CO_2 mixture, but they differed from Henderson & Haggard in that they found the fall in blood CO less rapid in a dog breathing air + CO_2 than in the dog breathing oxygen. Walton and co-workers (Walton, Eldridge, Allen & Witherspoon, 1926) used a larger number of dogs, ten in each group, and compared the rate of fall of blood CO in dogs breathing air, oxygen, and oxygen + 5% CO_2. Their mean figures show a more rapid fall of blood CO in the group breathing $O_2 + CO_2$ than in those breathing oxygen alone. They recommended the continued use of oxygen in resuscitation, on the grounds that it was more readily available and that it cleared the blood of CO 'almost as rapidly' as oxygen + CO_2. It is of interest that their figures show 10% CO_2 in air to be almost as effective as oxygen.

In more recent years Schwerma, Ivy & Friedman (1948) and others (Schwerma, Ivy, Friedman & La Brosse, 1948; Schwerma, Wolman, Sidwell & Ivy, 1948) have compared the effects of various methods of resuscitation on a large number of dogs poisoned by CO. Their object was to assess the value of various mechanical and manual methods of artificial respiration in addition to comparing the rate of fall of blood CO when oxygen or oxygen + 5 % CO_2 was used. Their experiments yielded a great deal of valuable information but the dogs breathed the various gas mixtures for only the first 4 min of the period of recovery and therefore no satisfactory conclusions can be drawn as to the relative merits of oxygen and an oxygen–CO_2 mixture.

It seemed desirable therefore to try to collect more data on this specific point. Dogs were chosen as the experimental animals because they are easy to handle, and because their blood has relative affinities for O_2 and CO very close to those of human blood. They have the further advantage that they have been extensively used by other workers in this type of investigation. This paper reports the results of allowing the dogs to breathe the various gas mixtures spontaneously; experiments using various methods of artificial respiration will be reported later.

METHODS

Fifty-nine dogs were exposed to mixtures of CO in air varying from 0·187 to 0·356 % CO, the concentration being kept constant during each exposure. Each dog was put into a steel box (Killick & Marchant, 1957) and was allowed to breathe air until temperature equilibrium was reached. Carbon dioxide was absorbed, as it was produced, by a tray of absorbent (Siebe Gorman 'Protosorb') placed under a fan in the top of the box. Oxygen entered the box, to replace the CO_2, through a Müller valve. The oxygen was passed through a rotameter to a small rubber bag, whence it bubbled through the valve into the box. The bag allowed for variations in the rate of oxygen consumption, and the rotameter reading provided a rough estimate of this rate. When the oxygen was bubbling in steadily it was assumed that temperature equilibrium had been reached, and a measured volume of CO was passed into the box through the rubber bag and Müller valve. The oxygen supply was then reattached.

The rise of temperature in the box and the addition of the CO caused a fall in the oxygen concentration of the mixture in the box. Gas samples were taken from the box after it was judged that a considerable proportion of the added CO had been absorbed by the dog; the samples were analysed for oxygen content in the Haldane gas-analysis apparatus, and for CO content by the Graham–Lawrence iodine pentoxide method (Haldane & Graham, 1935). Some time was occupied in passing the CO into the box; zero time was taken as the mid point between the time when the measured volume of CO was passed into the rubber bag and the time when all the CO had passed into the box.

Samples for oxygen analysis were taken at a time when oxygen was bubbling into the box at a regular rate. The times varied from 16 to 70 min, but the oxygen varied only from 18·4 to 19·7 % with a mean of 19·1 %. After the first 10–15 min the oxygen concentration remained steady; repeated analyses during several experiments showed slight variation, but no steady fall, and further the oxygen concentration found during the 59 exposures shows no correlation with the time of sampling.

Samples for CO analysis were taken after the period of most rapid absorption and after the dog was unconscious, usually at about 50 min, in order to obtain a value as near as possible to the concentration at the end of the exposure.

During exposure the dogs were observed, the time of onset of symptoms was noted, and the frequency of respiration was counted. Exposures varied in duration from 34 to 101 min, depending upon the time of onset of symptoms of severe poisoning. The purpose was to continue the exposure until the dog was deeply unconscious, and was on the verge of respiratory failure; the latter stage was judged by a slowing of respiration, and by a diminished inflow of oxygen to the box.

On removal from the box the dog was fitted with a mask (Chambers, Brewer, Davenport & Goldschmidt, 1946) and breathing valves, and connected to a Tissot spirometer containing 100% O_2, or 5% CO_2 in oxygen, or room air. The dog breathed the chosen gas mixture for 60 min, the volume inspired being recorded on a smoked drum by a writing point attached to the spirometer.

During this period of resuscitation capillary blood samples were taken from the ear for the determination of CO content by the method of Scholander & Roughton (1943). The first sample was taken as soon as possible after removing the dog from the box, and succeeding samples were taken at intervals of 5–15 min. Capillary blood was also used for the spectrophotometric determination of haemoglobin, for lactate determination (Barker & Summerson, 1941) and for measurement of pH, using a micro-electrode (Joels & MacNaughton, 1957). Some blood samples were taken from the femoral artery, and used for the direct measurement of CO_2 tension (Riley, Proemmel & Franke, 1945) and for the determination of plasma CO_2 content (van Slyke & Neill, 1924).

In rather more than half the experiments expired air was collected and analysed for CO_2, O_2 and CO. Expired air was pumped from the tube connecting the breathing valves with the Douglas bag and drawn through an infra-red meter at a rate of 400–450 ml./min; this provided a minute-by-minute record of the concentration of CO in the expired air. The dogs used in these experiments had their blood volumes determined on the day before exposure to CO. Evans Blue was used (Nitshe & Cohen, 1947); its concentration in the blood plasma was determined spectrophotometrically in a venous blood sample taken 5 min after the injection of a weighed volume of dye solution. Several experiments in which the dye concentration was determined at various intervals of time after injection showed that 5 min was the best time.

RESULTS

Symptoms during exposure to CO

The commonest first sign of poisoning was unsteadiness or stiffness of the legs as the dog walked; very often the animal lost its balance and fell. Some indication of ataxia was the first sign in thirty-eight of the fifty-nine dogs. In nineteen dogs vomiting was the first sign of poisoning, and it occurred in many of the other dogs subsequent to ataxic symptoms.

Most of the dogs exhibited a marked increase in frequency of respiration, the increase becoming evident after 10–20 min exposure to the CO in air mixture; in fourteen dogs the frequency was more than 100 respirations per minute. Five of these dogs, however, were observed to be panting (frequency of respiration of 100 breaths/min or more) during the first 10 min of exposure. During the period of exposure from 10 to 30 min the mean frequency of respiration was 61 breaths/min; only six of the fifty-nine dogs failed to show this increased frequency. After 30 min exposure there was a tendency for the frequency to drop; the mean frequency fell to 46 between 40 and 60 min exposure. Individual dogs exhibited marked variations in frequency during this period. After falling or lying down the dogs appeared gradually to become drowsy, and eventually lost consciousness; a dog was judged to be unconscious when it made no response to tapping on the side of the box.

The time of onset of these various signs and symptoms varied with the concentration of CO in the air, and also with the degree of activity and the frequency of breathing of the dog. In spite of the variation caused by these last two factors there does appear to be the expected relationship between concentration of CO and time of onset of symptoms, as Table 1 shows.

The total duration of exposure was not kept constant, since the object was to bring the dogs to a constant clinical state, verging on respiratory failure. No satisfactory single criterion of this state was found. The gasping described by Schwerma, Ivy & Friedman (1948) was very rarely observed; some dogs died without showing gasping respirations, and other survived for long periods after a few gasping respirations.

TABLE 1. Relation of symptoms to concentration of carbon monoxide

		Duration of exposure when symptom appeared (min)								
		Vomiting			Ataxia			Unconsciousness		
No. of dogs	Range of CO concentration (%)	Mean	max.	min.	Mean	max.	min.	Mean	max.	min.
10	0·30–0·356	20	29	12	18	29	12	29	49	21
7	0·275–0·295	25	38	13	20	36	7	28	59	16
10	0·26–0·274	24	43	16	24	42	10	35	58	20
12	0·24–0·259	27	55	16	22	33	14	35	54	19
10	0·225–0·238	32	45	14	34	54	19	42	68	20
10	0·187–0·223	28	49	15	28	45	15	40	62	19

Each dog was left in the box for a period of approximately 30 min after losing consciousness, unless signs of impending respiratory failure were detected earlier than this. Such signs were marked or progressive slowing of breathing, onset of periodic breathing, very shallow breathing, or a marked decrease in the rate at which oxygen entered the box, since this was taken to indicate a diminished rate of oxygen consumption.

Condition at end of exposure to CO. Four dogs were dead when removed from the exposure box. One of these suddenly stopped breathing after 17 min of unconsciousness, the other three were taken from the box after 27–32 min of unconsciousness because the breathing had become very slow; none of them breathed after being put on the operating table. In each case manual artificial respiration failed. Arterial blood taken post-mortem showed the haemoglobulin to be 86, 78, 81·2 and 87·5% combined with CO.

A fifth dog died while breathing 0·24% CO in air through a mask. This dog was exposed as usual in the box and taken out after 26 min unconsciousness; the mask was fitted on and connected to 0·24% CO-in-air in a Tissot spirometer. The breathing was very slow but did not seem to be decreasing when it just ceased. The mask was taken off and immediate manual artificial respiration applied but the dog was dead. Post-mortem blood sampling showed 77·5% of the haemoglobin to be combined with CO.

Three dogs died within 2 min of removal from the box. One breathed air for 80 sec and then stopped breathing; the other two took two or three breaths, but breathing ceased before the mask was put on. In each case manual artificial respiration failed. Blood taken post-mortem showed 72, 72·5 and 76·5% of the haemoglobin combined with CO.

One dog breathed 5% CO_2 +95% O_2 for 11 min and died with only 41% of the haemoglobin combined with CO. Another dog breathed the same mixture for 5 min and then died. The remaining forty-nine dogs survived the exposure with varying degrees of recovery; nine of these dogs breathed room air, twenty breathed 100% O_2 and the remaining twenty breathed 5% CO_2+95% O_2.

TABLE 2. Results of exposure to CO in air

		Condition of dogs				Recovered		
% COHb	No. of dogs	Dead	Died	Time of death	Mixture breathed	Air	O_2	CO_2+O_2
80 and over	9	3	—	—	—	—	2	4
75–79·9	20	2	2	30 sec 4 min	Air CO_2+O_2	3	7	6
70–74·9	28	—	3	30 sec 80 sec 11 min	Air Air CO_2+O_2	5	11	9
Below 70	2	—	—	—	—	1	—	1
Mean COHb (%)		82·0	74·9	—	—	72·2	75·1	74·8
Mean COHb (%) (excluding 2 dogs below 70%)		82·0	74·9	—	—	73·1	75·1	75·5

Table 2 classifies all fifty-nine dogs on the basis of the proportion of haemoglobin combined with CO at the end of exposure. The proportion of haemoglobin combined with CO is not a completely satisfactory criterion of the extent of hypoxia, since the haemoglobin content of the blood of these dogs varied from 11·2 to 21·5 g/100 ml. blood. If it is assumed that the haemoglobin not combined with CO is combined with oxygen in the arterial blood, the oxygen content of this blood at the end of exposure to CO may be calculated.

The five dogs which were dead on removal had a mean calculated arterial O_2 of 4·2 ml./100 ml. blood (2·6–5·8 ml.). The five dogs that died either within a minute of exposure, or during the first 12 min of attempted resuscitation, had a mean calculated arterial O_2 of 5·1 ml./100 ml. blood (4·4–6·2 ml.). The remaining forty-nine dogs had a mean arterial O_2 of 6·1 ml./100 ml. blood (3·8–9·8 ml.).

Measurement of pH and determination of lactate were made on the arterial blood of some of the dogs that died. Arterial blood, taken from the left heart or from an artery after death, was used for plasma CO_2 determinations, and in some cases for measurement of pH and of tension of CO_2. The results are shown in Table 3; although the figures are few they are interesting by reason of the low CO_2 content and partial pressure.

Dogs resuscitated

Dogs resuscitated by breathing air. The mean proportion of haemoglobin combined with CO for these nine dogs at the end of exposure was 72·2% (65–77). Two of them breathed less than 300 ml./kg body weight during the first minute of breathing air; the mean volume breathed by seven dogs for which the figures were complete is shown in Table 4.

Table 3. Dogs dead after exposure to CO

Determinations on arterial blood

Dog no.	% COHb	Hb (g/100 ml.)	Lactate (mg/ 100 ml.)	pH		Plasma CO_2 (ml./ 100 ml.)	pCO_2 (mm Hg)	
				m	*c*		*m*	*c*
				Dogs dead at end of exposure				
19	86	19·3	—	—	—	—	—	—
33	78	19·7	85	7·30	—	14·8	—	14
50	81·2	18·5	141·5	7·17	7·13	27·1	36	33
81	87·5	15·7	112	—	7·05	12·7	20·2	—
92	77·5	15·5	154	—	6·90	14·7	32·4	—
				Dogs died within 2 min of end of exposure				
27	72	16·4	—	7·11	—	8·8	—	13·5
49	72·5	12·6	106	7·05	—	—	48	—
55	76·5	14·0	126	—	—	—	21·4	—
				Dogs died within 12 min of end of exposure				
14	41	16·4	—	—	—	—	—	—
56	57·8	19·2	119	—	7·05	18·9	30	—

m = measured; *c* = calculated

Table 4. Volume of ventilation during recovery

No. of dogs	Mixture breathed		Volume inspired (ml./kg body wt./min) 0–1min	1–5 min	5–10 min	10–20 min	20–30 min	30–45 min	45–60 min
7	Air	Mean	421	521	425	363	342	380	417
		Range	802	995	720	662	516	653	760
			158	286	193	210	233	232	175
17	100% O_2	Mean	308	425	407	348	336	350	403
		Range	760	692	670	670	684	812	820
			53	144	109	111	130	148	115
17	$CO_2 + O_2$	Mean	388	792	961	990	1007	955	947
		Range	598	1420	1430	2150	2125	1925	1680
			27	27	219	392	538	570	577

In order to compare the rates at which CO disappeared from the blood in the dogs breathing different gas mixtures, 70% COHb was taken as the starting point and times were measured from this zero time. One of the dogs breathing air was excluded from these measurements, since its COHb was below 70% when it was first determined. The mean values for the remaining eight animals are shown in Fig. 1 and Table 5. The blood volume of five of these dogs was determined, and the expired CO was measured. Taking zero time as the time when the circulating COHb was 70%, the volume of CO lost

TABLE 5. % COHb during resuscitation: zero time 70% COHb

No. of dog	3 min breathing			9 min breathing			15 min breathing			25 min breathing			35 min breathing			55 min breathing		
	Air	O_2	O_2+CO_2	Air	O_2	O_2+CO_2	Air	O_2	O_2+CO_2	Air	O_2	O_2+CO_2	Air	O_2	O_2+CO_2	Air	O_2	O_2+C
15	—	61·8	—	—	49·3	—	—	39·8	—	—	31·2	—	—	25·5	—	—	18	—
16	—	—	52	—	—	36	—	—	28·3	—	—	19·8	—	—	14·5	—	—	7·4
17	—	54·5	—	—	42·2	—	—	34·1	—	—	24·1	—	—	17·7	—	—	13·3	—
20	—	—	50·5	—	—	31·7	—	—	24	—	—	16·5	—	—	11·5	—	—	7·5
21	—	50·7	—	—	37·2	—	—	27·0	—	—	17·7	—	—	13·2	—	—	6·8	—
22	—	61	—	—	47·5	—	—	37·3	—	—	26	—	—	19·3	—	—	12·1	—
23	65·3	—	—	58·5	—	—	54·2	—	—	48·8	—	—	45	—	—	39	—	—
26	—	—	54·8	—	—	36	—	—	25	—	—	15·5	—	—	10·0	—	—	7·0
28	65·7	—	—	58·5	—	—	52·5	—	—	45·5	—	—	41·3	—	—	34·8	—	—
30	—	58·7	—	—	45·8	—	—	36·6	—	—	28·1	—	—	23	—	—	15·2	—
31	—	—	59·8	—	—	45·5	—	—	34·5	—	—	21·8	—	—	15·0	—	—	8·9
32	64·5	—	—	58·5	—	—	54	—	—	48·8	—	—	43	—	—	35·2	—	—
34	—	63·9	—	—	55·2	—	—	47·8	—	—	37·3	—	—	32·2	—	—	24·2	—
35	—	—	52	—	—	38·4	—	—	30·3	—	—	21·2	—	—	15·8	—	—	7·7
40	—	62·8	—	—	53·3	—	—	45	—	—	36·7	—	—	31	—	—	22·2	—
41	—	—	63·3	—	—	51·6	—	—	41·8	—	—	27	—	—	21·3	—	—	13·8
42	—	—	58·6	—	—	44	—	—	32·8	—	—	24·8	—	—	19·5	—	—	14·0
43	—	63·8	—	—	55·1	—	—	47·7	—	—	35·2	—	—	30	—	—	20·8	—
44	—	65	—	—	57	—	—	49·8	—	—	40	—	—	33·7	—	—	—	—
45	—	64·8	—	—	54·8	—	—	46·4	—	—	35	—	—	27	—	—	16·8	—
46	64·8	—	—	58·8	—	—	54	—	—	45·7	—	—	40·2	—	—	32·7	—	—
47	—	—	49·7	—	—	34·4	—	—	24·2	—	—	22·7	—	—	9·0	—	—	6·0
48	—	—	55·5	—	—	40·4	—	—	29	—	—	18·8	—	—	11·8	—	—	8·0
51	—	—	58·5	—	—	39	—	—	25	—	—	15·3	—	—	9·6	—	—	5·9
54	—	63·7	—	—	52	—	—	43·5	—	—	31	—	—	24	—	—	17·5	—

TABLE 5 (*cont.*)

No. of dog	3 min breathing			9 min breathing			15 min breathing			25 min breathing			35 min breathing			55 min breathing		
	Air	O_2	O_2+CO_2	Air	O_2	O_2+CO_2	Air	O_2	O_2+CO_2	Air	O_2	O_2+CO_2	Air	O_2	O_2+CO_2	Air	O_2	O_2+CO_2
57	—	—	55	—	—	37·8	—	—	25·5	—	—	16·5	—	—	10·5	—	—	6·3
59	—	63·7	—	—	53·2	—	—	45·8	—	—	34·2	—	—	24·5	—	—	14·7	—
60	—	—	55·5	—	—	37·8	—	—	27·5	—	—	20·3	—	—	15·3	—	—	8·0
61	—	62·7	—	—	51·5	—	—	42	—	—	30·5	—	—	21·5	—	—	14·2	—
76	—	59·5	—	—	46	—	—	34·7	—	—	20·5	—	—	15·3	—	—	12·3	—
77	—	—	57	—	—	36·5	—	—	22·5	—	—	11·5	—	—	7·0	—	—	3·0
78	66·8	—	—	62	—	—	57·7	—	—	51	—	—	45·3	—	—	37·5	—	—
79	67	—	—	60·7	—	—	55·4	—	—	48	—	—	42·2	—	—	34·5	—	—
80	64·7	—	—	57·2	—	—	53·5	—	—	48	—	—	40·8	—	—	30·8	—	—
82	—	58·7	—	—	45·3	—	—	36	—	—	25·5	—	—	20·5	—	—	13·5	—
83	—	—	56·5	—	—	40·3	—	—	31·2	—	—	19·5	—	—	13·0	—	—	6·2
84	—	—	60·3	—	—	45	—	—	33·2	—	—	16·2	—	—	11·0	—	—	5·8
85	—	63	—	—	51	—	—	43·5	—	—	33·7	—	—	27	—	—	18·8	—
86	—	—	59·3	—	—	42	—	—	31·5	—	—	21	—	—	15	—	—	7·5
87	—	60·7	—	—	50	—	—	40·6	—	—	28·5	—	—	20·3	—	—	13·4	—
88	—	62·5	—	—	51	—	—	43·8	—	—	34·5	—	—	27·5	—	—	15·9	—
89	—	62·7	—	—	51·5	—	—	42·3	—	—	31	—	—	25·8	—	—	18	—
91	—	—	56·7	—	—	42·5	—	—	33·7	—	—	24	—	—	18·5	—	—	11·8
95	67·5	—	—	63	—	—	59	—	—	53·5	—	—	48·7	—	—	41·2	—	—
Mean	65·8	61·3	56·2	59·7	49·9	39·9	55·0	41·2	29·4	48·7	30·6	19·5	43·3	24·2	13·4	35·7	16·0	7·9
S.D.	1·16	4·33	3·68	2·02	4·97	4·83	2·22	5·78	5·15	2·64	5·84	4·05	2·86	5·62	3·94	3·39	4·11	2·86
t		3·76			6·07			6·41			6·5			6·51			6·75	
P		<0·001			<0·001			<0·001			<0·001			0·001			0·001	

from the blood and the volume of CO expired can both be expressed as a percentage of the total blood CO at zero time. The values obtained are shown in Table 6.

The rate of disappearance of CO from the blood may also be expressed as the volume of blood cleared of CO per minute (Apthorp, Bates, Marshall & Mendel, 1958). This value was calculated for each dog for which figures were available, for a period of 10 min during the first 15 min of resuscitation; the results are shown in Table 7 and Fig. 2.

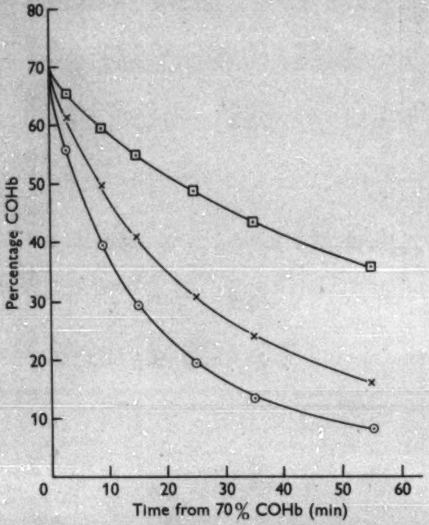

Fig. 1. Loss of CO from the blood during recovery. ⊡ % COHb in dogs breathing air (mean of eight dogs); × % COHb in dogs breathing 100% O_2 (mean of nineteen dogs); ⊙ % COHb in dogs breathing 5% CO_2 + 95% O_2 (mean of seventeen dogs).

Readings of the concentration of CO in the expired air were taken from the infra-red meter at approximately 15 sec intervals during the first hour of resuscitation. When these readings are plotted against time, the graphs for five dogs show a fairly regular pattern; the concentration of CO was never high, 0·07% was the highest reading even during the first few minutes. The concentration usually fell rapidly during the first 5 min and thereafter more gradually, until at the end of an hour it was in the region of 0·01%. The CO content (measured by the iodine pentoxide method) of expired air collected for approximately 15 min periods was also determined; the mean values for these five dogs are shown in Table 8. The volume of pulmonary ventilation

TABLE 6. CO lost from blood and CO recovered in expired air

Dog no.	Loss from blood as % total at 70% COHb				Recovered in expired air as % total at 70% COHb			
	+ 10 min	+ 20 min	+ 40 min	+ 55 min	+ 10 min	+ 20 min	+ 40 min	+ 55 min
				Dogs breathing air				
78	11·9	22·4	38	45·2	2·6	5·3	10·9	16·1
79	14	27·5	46·3	53·3	4·2	7·5	12·9	15·8
80	19·3	28·4	46·4	58·5	6·3	12·3	22·8	30·2
94	17·5	29	41·2	47·6	6·2	10·8	17·8	22·8
95	15·8	25·2	35·6	43·6	7·8	13·5	19·4	21·9
Mean	15·7	26·5	41·5	49·6	5·5	9·9	16·8	21·4
				Dogs breathing 100% O_2				
54	26·6	48·2	68·7	73·1	23·3	36·2	47·9	53
59	27·1	47·4	68·4	77·5	12	23·4	33·5	36·5
61	30·1	49·1	73	81·5	28·9	41	56·2	60·5
76	34·8	58·3	77·5	81·6	19·2	42·1	54·5	58·6
82	35	55·7	79·4	82·7	20·8	29·2	37	39·6
85	29·9	51	65·8	73·5	14·2	22	33·2	36·2
87	25·7	46·1	67·5	74	17·2	25·7	31·4	31·4
88	23·8	45·9	69·3	81·5	17·9	28·9	42·7	52·3
89	30·5	49·5	67·3	76·2	25·8	35·8	48·2	51·6
Mean	29·3	50·1	70·8	78·5	19·9	31·6	42·7	46·6
				Dogs breathing 5% CO_2 + 95% O_2				
57	48·7	76·2	89·7	92·2	38·5	52	61·2	61·2
58	42·3	69·7	83·6	88	33	47	58·3	61·8
60	51	70·2	83·5	90	28·1	37	44·4	46·8
75	36·3	59·2	80	85·8	19·5	32·2	45·3	51
77	51	80·8	94·5	98	27·5	44·5	61	65
83	45·9	65·7	83·2	89·5	15·3	24·3	31	31·5
86	44·6	66·6	82	89·4	30·2	46·7	60·4	67
91	44·3	61·5	77	83·4	29·8	43·7	59	64
Mean	45·5	68·7	84·2	89·5	27·7	40·9	52·6	56·0

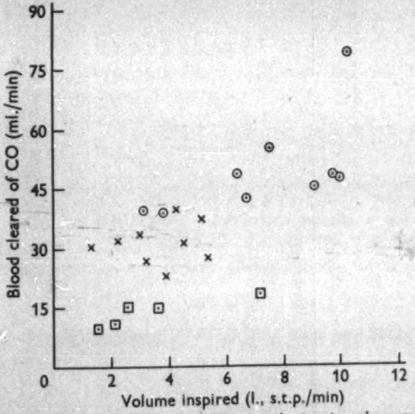

Fig. 2. Volume of blood completely cleared of CO per minute at various ventilation volumes; each point is the mean value during the first 15 min of the recovery. ⊡ dogs breathing air; × dogs breathing 100% O_2; ⊙, dogs breathing 5% CO_2 + 95% O_2.

Table 7. Volume of blood cleared of CO during first 15 min resuscitation

Breathing air				Breathing 100% O_2				Breathing 5% CO_2 + 95% O_2			
	Blood cleared				Blood cleared				Blood cleared		
No. of dog	(ml./min)	% blood vol./min	Insp. vol. (l./min)	No. of dog	(ml./min)	% blood vol./min	Insp. vol. (l./min)	No. of dog	(ml./min)	% blood vol./min	Insp. vol. (l./min)
78	15·3	1·2	2·55	54	32·4	3·4	2·19	57	49·2	6·1	9·72
79	10·0	1·6	1·54	59	30·8	3·3	1·32	58	48·7	5·5	6·37
80	18·6	1·6	7·21	61	34·2	3·7	2·96	60	80·0	6·7	10·20
94	15·0	1·6	3·61	76	31·6	4·3	4·48	77	39·2	6·6	3·10
95	10·9	1·3	2·09	82	37·7	4·2	5·09	83	38·8	6·0	3·76
				85	27·0	3·0	3·19	84	36·3	5·1	5·98
				87	40·0	3·8	4·21	86	43·0	5·5	6·66
				88	23·0	3·1	3·91	90	48·0	5·0	9·90
				89	28·0	3·2	5·38	91	56·0	5·4	7·47
								93	46·0	4·7	9·03
Mean	14·0	1·5	3·40	—	31·6	3·6	3·64	—	48·5	5·7	7·22

during resuscitation is summarized in Table 4, and is shown related to the rate of fall of blood CO in Fig. 3.

During the hour that the dogs breathed from the Tissot spirometer signs of recovery were noted, and the condition at the end of this period was also noted. The dogs regained consciousness at times varying between 7 and 30 min, mean 16·4 min. The % COHb at the time of recovering consciousness varied between 47·2 and 62·6, mean 55·8. Five dogs sat up during this first hour, at times varying between 23 and 58 min. The other four dogs made no attempt to sit up (Table 9). At the end of the hour the dogs were put on the

TABLE 8. CO in expired air

No. of dogs	Mixture breathed		% CO in collected expired air during recovery			
			0–15 min	15–30 min	30–45 min	45–60 min
5	Air	Mean	0·024	0·018	0·014	0·011
		Range	0·034 0·014	0·021 0·013	0·017 0·013	0·013 0·009
6	100% O_2	Mean	0·053	0·026	0·013	0·006
		Range	0·086 0·035	0·034 0·015	0·021 0·003	0·013 0
9	5% CO_2 + 95% O_2	Mean	0·054	0·018	0·008	0·003
		Range	0·070 0·026	0·028 0·007	0·017 0·001	0·010 0

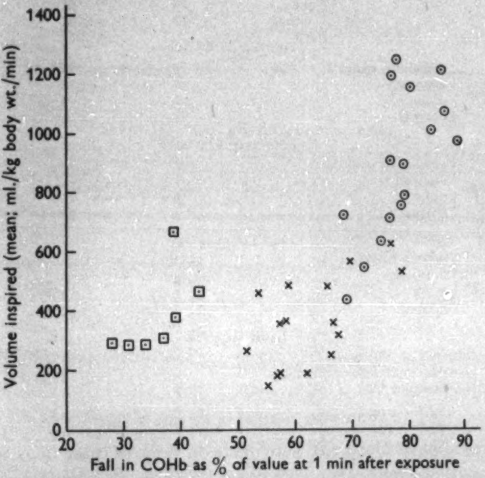

Fig. 3. Fall in blood CO in relation to volume of ventilation during period 1 min to 30 min of recovery. ⊡ dogs breathing air; × dogs breathing 100% O_2; ⊙ dogs breathing 5% CO_2 + 95% O_2.

floor and left to their own devices; at this stage seven dogs could walk, though all were unsteady and tended to fall. The other two dogs were quite unable to stand. Three or four hours later six of the dogs could walk steadily, but were disinclined to move, one still moved very stiffly, and the remaining two were still unable to stand. On the day after exposure six dogs appeared normal, one moved normally but was very bad-tempered, one was very quiet, and one still moved stiffly. Within a week all the dogs appeared normal. One of them gave birth to five pups 6 weeks after exposure.

TABLE 9. Stages of recovery

Mixture breathed ...		Air	100% O_2	5% CO_2 + 95% O_2
No. of dogs		9	20	19
Time of regaining consciousness	Mean	16·4	9·5	8·3
	max.	30	23	21
	min.	7	2	1
No. of dogs sitting up		5	13	16
Time of sitting up	Mean	41	29·4	33·8
	max.	59	50	50
	min.	23	12	13·5
Condition at 60 min				
No. of dogs	unable to stand	2	1*	1
	unsteady	7	6	4
	quiet	—	4	4
	normal	—	9	11
Condition 3–4 hr later				
No. of dogs	unable to stand	2	—	1
	unsteady	1	—	—
	quiet	6	10	4
	normal	—	10	14
Condition next day				
No. of dogs	unable to stand	—	—	1
	unsteady	1	—	—
	quiet	1	3	—
	normal	7	17	18
Later sequelae				
Cases of	illness? pneumonia	—	4	2
	ataxia, weakness or paralysis	—	2	2
	bad temper	1	1	—

* This dog breathed O_2 for only 30 min.

Determinations of blood pH were made on three of these dogs, and of blood lactate on five of them. Capillary blood for these determinations was taken at convenient times during resuscitation; it proved impossible to adhere to a rigid programme, and the times of sampling therefore differed for each dog, In order to facilitate comparison, the values for pH and for blood lactate were plotted against time from the end of exposure for each dog. Values at fixed times were then read from the graphs so constructed. These values for the dogs breathing air are summarized in Tables 10 and 11. In order to make a comparison of the values easier, these dogs with a blood lactate below 55 mg/100 ml. blood at 5 min after exposure to CO have been excluded from the means shown in Fig. 4; this makes it obvious that the blood lactate fell more slowly in the dogs breathing air than in those breathing O_2 or O_2+CO_2.

Blood samples were taken from the femoral artery in five of these dogs plasma CO_2 content was determined (van Slyke & Neill, 1924) in every sample, pH was determined in some samples, and CO_2 tension was determined by the bubble method (Riley *et al.* 1945) in some samples. The results are shown in Table 12.

TABLE 10. Blood lactate during resuscitation

No. of dogs	Treatment		mg lactate/100 ml. blood Time from end of exposure to CO (min) 5	15	25	40	55
5	Air	Mean	84·3	74·8	65·6	53·7	43·7
		Range	97	86·5	75·7	62·2	53·8
			69·5	62·5	55·5	42·7	25
15	100% O_2	Mean	77·1	66·9	56·7	42·1	29·9
		Range	113·7	103	94·5	77·5	61
			39	32·8	23·5	18	10·5
		Mean of values 55 and over	84·6	73·7	62·4	46·3	33·2
17	5% CO_2 + 95% O_2	Mean	71·2	60·3	50·7	38·3	25·5
		Range	150	128·5	109·5	95·2	79·5
			32·2	19	16	12	5·2
		Mean of values 55 and over	79·3	65·9	53·9	38·4	22·5

TABLE 11. Blood pH during resuscitation

Dog No.	Mixture breathed*	Time after end of exposure to CO (min) 5	10	15	25	40	55	75
23	Air	7·26	7·26	7·27	7·27	7·29	7·32	7·36
32		7·23	7·23	7·27	7·30	7·34	7·38	7·40
46		7·23	7·21	7·23	7·28	7·29	7·30	7·34
Mean		7·24	7·23	7·26	7·28	7·31	7·33	7·37
22	100% O_2	7·21	7·21	7·21	7·27	7·34	7·37	7·40
30		7·30	7·28	7·27	7·27	7·32	7·32	7·41
34		7·31	7·32	7·33	7·33	7·36	7·41	7·44
40		7·20	7·20	7·21	7·24	7·35	7·37	7·40
43		7·24	7·25	7·21	7·26	7·32	7·39	7·45
44		7·19	7·18	7·19	7·25	7·31	7·34	—
Mean		7·24	7·24	7·24	7·27	7·33	7·37	7·42
26	5% CO_2 + 95% O_2	7·19	7·19	7·18	7·25	7·34	7·41	7·43
31		7·17	7·15	7·14	7·14	7·21	7·26	7·39
35		7·21	7·17	7·22	7·30	7·33	7·36	7·45
41		7·23	7·22	7·21	7·22	7·29	7·35	7·40
47		7·16	7·15	7·15	7·21	7·29	7·32	7·41
48		7·11	7·10	7·12	7·16	7·20	7·23	7·32
Mean		7·18	7·16	7·17	7·21	7·28	7·32	7·40

* After 60 min all dogs breathed air.

Dogs resuscitated by breathing 100% O_2. Twenty dogs survived the day of exposure after breathing 100% O_2 for 1 hr. The mean proportion of haemoglobin combined with CO, on removal from the box, was 75·1% (70–81·2).

One dog was rigid when lifted from the box, its ear failed to bleed when cut, and its breathing seemed weak. The ear of another dog failed to bleed when cut, and twelve other dogs were breathing feebly or irregularly when taken from the box; ten dogs breathed less than 300 ml./kg body weight during the first minute of O_2 breathing. Thereafter the volume breathed increased, but after 5–10 min it diminished again (see Table 4).

Blood determinations showed that the fall in CO content was much faster than in the dogs breathing air (Fig. 1), and the signs of recovery were noticed correspondingly earlier. The dogs recovered consciousness at times varying from 2 to 23 min with a mean of 9·5 min; at this time the % COHb varied from 39·7 to 72, with a mean of 56·0. Thirteen dogs sat up while breathing O_2, and three dogs sat up when the mask was taken off, after about 60 min breathing O_2 (see Table 9). The mean % COHb at the time of sitting up for these sixteen dogs was 29·4 (range 16·5–44·5).

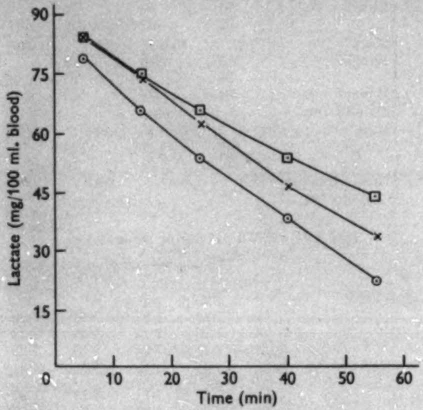

Fig. 4. Blood lactate values during recovery; mean values, excluding those below 55 mg lactate per 100 ml. blood at 5 min. ⊡ mean for five dogs breathing air; × mean for twelve dogs breathing 100% O_2; ⊙ mean for thirteen dogs breathing 5% CO_2 + 95% O_2.

When lifted from the table to the floor nine dogs ran about, apparently normal, four dogs could walk steadily but were very quiet and sluggish, six walked unsteadily; the remaining dog (no. 44) breathed O_2 for only 30 min, because it struggled and broke the valve holder; when put on the floor it was unable to stand. The same accident occurred with another dog (no. 53). This one is included among the nine dogs that appeared normal, although it breathed O_2 for only 30 min. On the fifth day after exposure, however, this dog became ataxic and possibly blind, since it seemed unable to avoid objects in its path;

TABLE 12. Arterial blood during resuscitation

Dog no.	Plasma CO$_2$ (ml./100 ml. blood)				pH								pCO$_2$ (mm Hg)							
	0–5 min	5–10 min	10–30 min	After 30 min	0–5 min		5–10 min		10–30 min		After 30 min		0–5 min		5–10 min		10–30 min		After 30 min	
					m	*c*	*m*	*c*	*m*	*c*	*m*	*c*	*m*	*c*	*m*	*c*	*m*	*c*	*m*	*c*
Dogs breathing air																				
32	—	22·2	31·0	—	—	—	7·23	—	7·32	—	—	—	—	—	—	23·5	—	27	—	—
46	26·7	—	31·5	—	7·24	7·18	—	—	7·24	7·26	—	—	32	28	—	—	31	32	—	—
80	20·2	—	24·4	—	—	7·04	—	—	—	6·98	—	—	33	—	—	—	45	—	—	—
94	—	26·2	—	—	—	—	—	—	—	—	—	—	—	—	49·5	—	—	—	—	—
95	—	—	32·1	—	—	—	—	—	—	7·04	—	—	—	—	—	—	52	—	—	—
Dogs breathing 100% O$_2$																				
40	—	22·3	28·5	35·7	—	—	7·20	—	7·21	—	7·37	—	—	—	—	25	—	31·5	—	28·5
43	28·5	—	—	—	7·22	—	—	—	7·23	—	—	—	—	31	—	—	—	—	—	—
44	—	25·8	32·5 32·8	—	—	—	7·19	—	7·20 7·28	—	—	—	—	—	—	30	—	36·5 32	—	—
82	18·8	—	31·2	—	—	7·11	—	—	—	7·20	—	—	25·8	—	—	—	35·6	—	—	—
85	—	—	32·7	—	—	—	—	—	—	7·12	—	—	—	—	—	—	44	—	—	—
87	—	—	21·5	—	—	—	—	—	—	6·94	—	—	—	—	—	—	42	—	—	—
Dogs breathing 5% CO$_2$+95% O$_2$																				
31	17·1	—	—	—	7·17	—	—	—	—	—	—	—	—	21	—	—	—	—	—	—
35	—	26·4	—	39·0	—	—	7·16	—	—	—	7·30	—	—	—	—	33	—	—	—	35
41	—	—	35·2	—	—	—	—	—	7·22	—	—	—	—	—	—	—	—	38	—	—
48	—	—	23·4 31·9	39·6	—	—	—	—	7·12 7·16	—	7·22	—	—	—	—	—	—	31 39	—	42
83	—	39·0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
84	—	—	29·2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
86	27·4	—	—	—	—	7·13	—	—	—	—	—	—	36·3	—	—	—	—	—	—	—

m = measured; *c* = calculated.

3 weeks after exposure this dog was still stiff and somewhat ataxic, and seemed to be blind. Gradually the gait improved until 3 months later the dog walked normally but still seemed blind. Dog no. 44 also suffered a relapse; it became weaker from the second day after exposure, and at the end of a week its legs appeared paralysed, and it was fed by hand. On the tenth day recovery began, and after 2 weeks the dog appeared normal again; it remained healthy for the next month and was then killed.

Thirteen dogs appeared normal on the day after exposure, and remained healthy. One dog became sluggish and bad-tempered a few days after exposure, and remained in this condition until killed 2 weeks later; four dogs developed pneumonia, one on the fourth day, one on the fifteenth day, and two after 3 weeks. Post-mortem examination showed patchy consolidation of the lungs in each case. It seemed doubtful whether the exposure to CO was a factor in causing the pneumonia, except possibly in the dog that developed it 4 days after exposure.

Determinations of blood pH were made on six of these dogs, and of blood lactate on fifteen dogs. The results were treated as were those for the dogs breathing air and are shown in Tables 10 and 11 and in Fig. 4.

The blood volume of nine of these dogs was determined, and the expired CO was measured. The mean values for loss of CO from the blood and for its recovery in the expired air are shown in Table 6. The highest readings for concentration of CO in the expired air (infra-red meter) were obtained with these dogs during the first 5 min of resuscitation; on several occasions the concentration was above the sensitivity range of the meter (0·25% CO) for brief periods. The peak of concentration was always reached during the first 5 min, and thereafter the concentration fell, rapidly at first and then more slowly, until low levels were reached before the end of the hour; in three of the nine dogs whose expired CO was metered the concentration fell too low to record (i.e. below 0·001%) during the last 10 min of the resuscitation period. The mean values for the concentration of CO (iodine pentoxide method) in the collected expired air are shown in Table 8.

Blood was taken from the femoral artery of six of these dogs; plasma CO_2 content was determined on each sample, pH was measured for some samples, and partial pressure of CO_2 was directly measured for some samples. The results are shown in Table 12.

Dogs resuscitated breathing 5% CO_2 + 95% O_2. Twenty dogs breathing this mixture survived the day of exposure. The % COHb at the beginning of resuscitation varied from 81·5 to 61·5, with a mean of 74·8. Only one dog had COHb below 70%; this dog was taken from the box because breathing ceased, but manual artificial respiration restarted the breathing. Since the haemoglobin was only 61·5% combined with CO, this animal has been excluded from the analyses of results, because zero time has been taken as the time when the haemoglobin was 70 % combined with CO. Omitting this dog from the figures given above, the range at the beginning of resuscitation would become 70–81·5 % COHb, with a mean of 75·5 %.

Of these nineteen dogs, two had ceased breathing when removed from the box; one of these started to breathe again spontaneously, the other needed manual artificial respiration for about 3 min before spontaneous breathing recommenced. One dog was rigid when taken from the box, and its ear failed to bleed when cut. In two other dogs the ear failed to bleed, and these three together with eleven other dogs were breathing feebly, either irregularly or slowly. Six of these dogs breathed less than 300 ml./kg body weight during the first minute, but thereafter the volume of breathing increased markedly, and remained high for the remainder of the 60 min breathing $CO_2 + O_2$ (Table 4). The fall in blood CO was even faster in these dogs than in the group breathing O_2 alone (Table 5 and Fig. 1). though the signs of recovery appeared very little earlier (Table 9). It was often difficult to judge when the dogs regained consciousness, but for the eleven dogs in which the time was definite it varied from 1 to 21 min with a mean of 8·3 min. At the time of regaining consciousness COHb varied from 20·3 to 67 %, with a mean of 49·2 %.

Sixteen of the nineteen dogs sat up during the first 60 min, at times varying from 13·5 to 50 min, mean 33·8 min; at this time the COHb varied from 8 to 30·7 %, mean 15·7 %. Two dogs sat up as the mask was taken off, but the remaining dog did not move at this time. This last dog was the only one of the group that failed to recover. Some 4 hr after exposure the dog made an attempt to stand, but was extremely unsteady. On the following day the dog was roused with difficulty, and walked unsteadily when it could be induced to try. The dog's condition remained much the same, the ataxia becoming slightly worse; it ate badly and lost 6 lb. (2·7 kg) in weight. It was killed on the thirteenth day.

Of the other dogs in this group eleven seemed quite normal at the end of the hour breathing $CO_2 + O_2$, four could walk normally but were unusually quiet, four ran about but were somewhat unsteady. Later in the same day fourteen of the dogs seemed normal, four were still quiet, and one was somewhat unsteady, but all were normal the following morning. Four days later one dog developed ataxia; this progressed until on the twelfth day the dog was totally unresponsive, was not eating, and seemed slightly spastic in addition to the ataxia. Three days later, however, the dog was eating unaided, and could stand, although unsteadily. Recovery continued, although 3 weeks after exposure the dog became extremely excitable; this gradually passed off and 5–6 weeks after exposure recovery appeared to be complete. Two dogs became ill on the seventh and fourteenth days after exposure. One of these recovered completely in a week, the other became very weak and ceased to eat and was killed after 5 days' illness; both lungs showed patches of consolidation. One of these dogs gave birth to two pups, one of which was still-born, 3 weeks after exposure.

Determinations of blood pH were made on seven of these dogs; the results are shown in Table 11. Blood volume was determined and expired CO measured in ten of the dogs; the calculated loss of CO from the blood, and recovery of CO in the expired air are shown in Table 6. The concentration of CO in the expired air was metered in ten of these dogs; in nine of them there was a peak concentration during the first 5 min, but the concentration reached was not higher than 0·13%; thereafter the concentration fell rapidly, reaching 0·01–0·015% at 30 min, and in five dogs the concentration was below 0·001% before the end of the hour. The tenth dog showed a peak concentration of expired CO resembling that of the dogs breathing O_2, exceeding 0·25% for 3 min during the first 5 min; thereafter the concentration fell very rapidly to 0·001% in 47 min. It is interesting that this animal had a low volume of ventilation during the first 5 min of breathing $CO_2 + O_2$, but during the seventh minute the volume of ventilation was more than trebled, and continued to increase.

The mean values for the CO content of the collected samples of expired air (iodine pentoxide method) are shown in Table 8. Blood lactate was determined in fifteen of these dogs, and the results are shown in Table 10 and Fig. 4. Blood was taken from the femoral artery in seven dogs; plasma CO_2 was determined on all the samples, pH of some samples was measured, and partial pressure of CO_2 was directly measured on some samples. The results are shown in Table 12.

DISCUSSION

The manner of exposing the dogs to CO precluded any quantitative study of the effects on breathing during exposure, but the change in frequency of respiration was a likely indication of increase in respiratory volume. Hyperpnoea during exposure to CO–air mixtures has been observed in the human subject when concentrations greater than 0·1% are breathed (Haldane, 1895), although the resting subject showed no significant increase in breathing when lower concentrations were used (Haldane, 1895), or when sufficient CO was inhaled to combine with about 40% of the circulating haemoglobin (Asmussen & Chiodi, 1941). Hyperpnoea during breathing CO–air mixtures has been recorded in dogs (Henderson & Haggard, 1920; Haggard & Henderson, 1921; Walton *et al.* 1926; Kamei, 1931*b*; Swann & Brucer, 1949; Lillehei, Wilks & Carter, 1954) and in rabbits (Mikami, 1927; Kamei, 1931*a*; Miura, 1936). Such evidence as is available suggests that this increased ventilation is unlikely to be due directly to the increasing proportion of haemoglobin combined with CO. The carotid and aortic chemoreceptors are known to respond to a fall in partial pressure of oxygen in the arterial blood; such a fall is unlikely to occur during absorption of CO, at least until respiratory failure begins.

The experiments of Duke, Green & Neil (1952) confirm that the combination of 75–80% of the haemoglobin with CO in anaesthetized cats elicits no response from the carotid chemoreceptors. Comroe & Schmidt (1938) recorded similar results in anaesthetized dogs. Blood containing approximately 8% (v/v) of CO caused no reflex hyperpnoea when perfused through the carotid body, provided the O_2 tension of the blood was within normal limits. A possible cause of the increased ventilation is a falling pH of the arterial blood; the pH has been shown to be low at the end of the period of exposure and one may presume that the fall was gradual during absorption. This lowered blood pH was associated, in these dogs, with a raised blood lactate content; it may well be that a rise in other metabolic acids accompanied the rise in lactate. This metabolic acidosis suggests that the tissue hypoxia results in a change in the normal metabolic pathway.

It would seem that the acidosis is partially compensated by a loss of CO_2 since the arterial samples taken early during resuscitation, or taken post-mortem from dogs dead at the end of exposure, showed a low plasma CO_2 content, and a low partial pressure of CO_2. Blood from two of the dogs dead at the end of exposure was equilibrated with air containing CO_2 at 40 mm partial pressure; the plasma CO_2 content was 15·8 and 25·4% (v/v) (7·1 and 11·4 mM) compared with a mean value of 45% (v/v) (20·2 mM) for three dogs killed more than a month after exposure to CO. These changes in arterial CO_2 resemble those found by Haggard & Henderson (1921) in dogs breathing CO–air mixtures, although they deduced an increase in blood pH. Kamei (1931*b*), also using dogs, observed reduced arterial CO_2 and a lowered pH during inhalation of similar mixtures. Swann & Brucer (1949) gassed four dogs with 1% CO in air, and during their rather brief survival time observed a fall in blood CO_2, a fall in blood pH, and a rise in blood lactate.

In connexion with this lowering of blood CO_2 and bicarbonate, it is interesting to note that there were some indications of retention of CO_2 during the first 15–30 min of resuscitation. Expired air was usually collected for 15 min periods, and in six of the seven dogs breathing CO_2 and O_2 for which complete analyses of expired air were made the CO_2 content of the expired air during the first 15 min was less than the 5% in the inspired air. In these dogs and in those breathing oxygen it was difficult to calculate a respiratory quotient, since at this stage N_2 was being washed out of the lungs. Complete analysis of expired air was done for four dogs breathing air, and of these three had low respiratory quotients during the first 15 or 30 min, the values being 0·64, 0·49, 0·61.

Although the blood pH was always low at the end of exposure to CO, stimulation of breathing was caused by 5% CO_2 in O_2, and this was associated with a rapid loss of CO from the blood. The rate of this loss was in proportion to the volume of ventilation, and no evidence was obtained that the addition of 5% CO_2 to the oxygen had any effect on the dissociation of CO from the haemoglobin, other than the indirect effect of the greater ventilation of the lungs. The high volume of ventilation was associated with a low concentration of CO in the expired air, and this presumably indicates a low concentration of CO in the alveolar air. Since the rate of dissociation of COHb depends on the partial pressures of oxygen and CO, it is clear that the increased ventilation hastened the loss of CO from the blood by lowering its partial pressure in the alveolar air.

It is obvious from Table 6 that during the first hour of resuscitation more CO disappeared from the blood than was recovered in the expired air; the discrepancy was greatest in the dogs breathing air, when the mean value for CO lost in the expired air during 55 min was only 43·2 % of the mean value for CO disappearing from the blood. The proportion of CO recovered in the expired air was greater in the dogs breathing oxygen and 5 % CO_2 in oxygen. The mean value for CO lost in the expired air was 59·7 % for dogs breathing oxygen and 62·7 % for dogs breathing 5 % CO_2 in oxygen, in each case as a percentage of the CO disappearing from the blood during 55 min. The difference between these two values is not statistically significant ($P = 0{\cdot}65$). We have no evidence, as yet, on the fate of the CO that disappears from the circulating blood but does not appear in the expired air. Our results, however, seem to agree reasonably well with those of Roughton & Root (1945), in experiments on man, although for obvious reasons the level of blood CO was very much lower in their experiments. These authors carried out similar measurements on dogs during haemorrhagic or traumatic shock, again using low blood CO contents; during 30 min periods the expired CO was 43–67 % of the volume of CO lost from the circulating blood.

It has often been assumed that when respiration is beginning to fail, as a result of hypoxia, there must be a retention of CO_2 in the body, and hence an increase in partial pressure of CO_2 in the arterial blood. Swann & Brucer (1949) poisoned dogs rapidly with 1 % CO in air, and found a falling partial pressure of CO_2 in the arterial blood during the 15–20 min that the dogs breathed, followed by a terminal rise when the dogs stopped breathing. After the slower poisoning of our experiments we found no such terminal rise, but the evidence cannot be regarded as conclusive. It seems possible that severe hypoxia might reduce the total metabolism and also produce some anaerobic form of metabolism that would not liberate CO_2.

The rate of recovery of the dogs may be assessed in various ways. If we consider the loss of CO from the blood, together with the loss from the whole body in the expired air, it is clear that CO was lost very much more slowly by the dogs breathing room air than by those breathing either 100 % O_2 or 5 % CO_2 + 95 % O_2. This is what one would expect, since the partial pressure of O_2 in the alveolar air must be much lower in the dogs breathing air. Comparing the dogs breathing 100 % O_2 with those breathing 5 % CO_2 + 95 % O_2, the

CO disappeared more rapidly from the circulating blood of the dogs breathing the CO_2 mixture ($P < 0{\cdot}001$ for all the times compared), but comparing the rate at which CO was lost in the expired air the difference is not so marked. A statistical analysis of the values given in Table 6 for these two groups of dogs yields a value for P of 0·04 after 10 min, 0·05 after 20 min, 0·09 after 40 min, and $> 0{\cdot}10$ after 55 min. The number of experiments is, however, small compared with those in which blood CO was determined; it is possible that a larger number would yield a more significant difference. It seems clear that at least as great a proportion of the CO lost from the blood appears in the expired air in dogs breathing $CO_2 + O_2$ as in dogs breathing 100% O_2.

During recovery the blood pH gradually rose to normal levels while the blood lactate fell. During the same period the arterial plasma CO_2 content rose, and the few measurements made suggest a corresponding rise in partial pressure of CO_2 in the arterial blood. All these changes proceeded more rapidly in the dogs breathing 100% O_2 or 5% CO_2 + 95% O_2 than in the dogs breathing air.

The fall in blood lactate proceeded at similar rates in the dogs breathing 100% O_2 and in those breathing 5% CO_2 + 95% O_2, although the small difference in slope of the curves in Fig. 4 suggests a slightly greater rate of disappearance in the dogs breathing $CO_2 + O_2$. This difference is supported by the few determinations of blood lactate made after the first hour of recovery; although at this time all the dogs were breathing room air, 7 out of 8 determinations of lactate on dogs that had breathed the CO_2 mixture were within the normal range (2–24 mg/100 ml.) and only 3 out of 7 determinations on dogs that had breathed 100% O_2 were within this range. These determinations were all made during the 30 min following the hour of breathing O_2 or $O_2 + CO_2$.

During the hour of oxygen breathing the pH of capillary blood was consistently somewhat higher in the dogs breathing 100% O_2 than in those breathing the CO_2 mixture. During the following 15 min, however, when all the dogs breathed air, the blood pH of the two groups of dogs became very similar. Comparing the clinical condition of the dogs during the recovery period (see Table 9) it is clear that recovery was more rapid when oxygen was breathed than when air was used throughout. Comparing the effects of 100% oxygen with those of the CO_2 mixture the differences were small, but complete recovery occurred a little earlier in the dogs that had breathed the CO_2 mixture. Later sequelae were few, and their incidence was similar in all three groups of dogs.

It would seem that when dogs are poisoned with CO at concentrations between 0·20 and 0·30%, the heart fails at the same time or earlier than the respiration. In the animals that ceased to breathe on the operating table no heart beat could be felt at this time, and manual artificial respiration failed. The only exception to this was dog no. 58; this dog ceased to breathe at the time the mask was put on and connected to the spirometer containing 5% CO_2+95% O_2. The heart beat was just palpable, and spontaneous respiration began after 2–3 min manual artificial respiration. Although many of the dogs might be said to be in a stage of failing respiration (i.e. slow, shallow, or irregular) at the end of exposure, this one dog was the only animal showing respiratory failure that survived.

It can be stated with certainty that in this series of experiments the CO disappeared significantly more rapidly from the circulating blood when the dogs breathed 5% CO_2 in oxygen than when they breathed oxygen alone. The CO was also eliminated correspondingly more rapidly in the expired air when $CO_2 + O_2$ was breathed. There was no evidence of a high partial pressure of CO_2 in the arterial blood, in spite of a diminished volume of breathing at the end of the period of exposure.

It should be noted that the only two dogs that died during the first 15 min of attempted resuscitation were breathing $CO_2 + O_2$; unfortunately arterial blood was examined in only one of them, and in this dog neither plasma CO_2 content nor partial pressure of CO_2 was raised. It seems doubtful, therefore, whether death can be attributed to breathing the CO_2 mixture.

SUMMARY

1. Dogs were exposed to air containing 0·2–0·3% carbon monoxide until they appeared to be near respiratory failure.

2. On removal from the carbon monoxide–air mixture the dogs breathed air, or 100% oxygen, or 5% carbon dioxide in oxygen.

3. Loss of carbon monoxide from the blood, and its elimination in expired air were markedly slower when air was breathed.

4. Loss of carbon monoxide from the blood was significantly faster when oxygen and carbon dioxide was breathed than with oxygen alone. This difference was related to a greater volume of ventilation when carbon dioxide plus oxygen was breathed.

5. During the first hour of recovery about 60% of the carbon monoxide lost from the blood appeared in the expired air of the dogs breathing oxygen or oxygen plus carbon dioxide. The expired carbon monoxide of dogs breathing air was 43% of the volume lost from the blood.

6. Blood lactate pH and plasma CO_2 content were determined during the recovery period.

It is a pleasure to express our gratitude to Dr Samueloff, who helped with the blood CO determinations in some of the early experiments, to Dr Joels and Mr MacNaughton, who made many of the blood pH determinations, and to Dr Beeching, who helped with the expired air analysis.

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MATURATION OF THE HYPOTHALAMO-NEUROHYPOPHYSIAL SYSTEM

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New-born animals (Heller, 1949) and infants (Hansen & Smith, 1952; Barnett & Vesterdal, 1953) are unable to concentrate their urine to the same extent as adults. The following hypotheses or their combinations have been considered (Heller, 1958) to explain this difference: (1) too little of the antidiuretic hormone (vasopressin) is produced, (2) the osmoreceptors do not respond or do not respond adequately to the stimulus of dehydration, (3) the renal tubules do not react to vasopressin in the adult manner, either because of morphological or functional immaturity, or because they are under the influence of other hormones which antagonize vasopressin.

The validity of the first hypothesis could be tested by comparing blood levels of vasopressin in hydropenic new-born and adult mammals, but the difficulties of estimating vasopressin in the blood of new-born animals have so far been unsurmountable. Several investigators have therefore approached this problem by estimating the hormone content of the neurohypophysis. In relation to body weight Heller (1947) found considerably less antidiuretic activity in the glands of rats aged 18–42 hr than in the pituitaries of adults. Dicker & Tyler (1953), who referred hormone content to body surface, reported that the glands of dogs, cats and guinea-pigs at birth contained about half the activity of adult glands. However, since formulae for the calculation of the body surface of the new-born of these species were not available, they based their results on Lee's (1929) formula for the body surface of adult rats. This may have influenced their conclusions. The neurohypophysis of new-born infants has also been found (Heller & Zaimis, 1949) to contain less antidiuretic and pressor activity than that of adults.

The results presented in this paper extend previous findings in new-born rats (Heller, 1947) by reporting on the hormone content of rat neurohypophyses throughout the first month after birth. Both pressor and oxytocic activities were determined. This seemed important since vasopressor:oxytocic ratios have been reported in infant animals (Dicker & Tyler, 1953; Acher, Chauvet & Olivry, 1956) which differ substantially from those of adults. A few guinea-pig and human glands were also investigated.

METHODS

Animals

Rats of both sexes aged from 1 to 28 days were used. Since the hormone content per gland appears to be related to body weight as well as to age, and body weight is known to be influenced by the number of young in the litter, litter size was uniformly restricted to six. Adult male rats of the same strain (Wistar) weighing 180–250 g served as controls.

Guinea-pigs aged about 24 hr and male adults of the same strain were used. Human pituitary glands were removed from 3 to 48 hr after death from bodies which had been kept in cold store until shortly before dissection.

Preparation of extracts

The rats were killed by decapitation. Whole pituitaries were extracted of animals aged up to 7 days, but neural lobes only of older animals. All glands were extracted immediately after the rats had been killed. Following the suggestion of Bentley & Dicker (1955) a number of neurohypophyses of infant rats of each age group were pooled for extraction (per extract, 18–24 glands were pooled of 1-day-old rats, 12–15 glands of 3–5-day-old rats, 6–9 glands of 7–14-day-old rats and 4–6 glands of 21–28-day-old rats). This allowed dilution of the acid extracts with the uterus suspension fluid (see below) to prevent decreases of pH which, as these authors have shown, may modify the response of the isolated uterus to oxytocin. Extracts for vasopressor assays were diluted with NaCl solution 0·9% (w/v).

Acetic acid extracts. The pooled glands, mixed with 0·4 ml. suspension fluid containing acetic acid 0·25% (v/v), were crushed in a small (3·5 ml.) glass homogenizer and transferred to a 15 ml. centrifuge tube, using a further 0·6 ml. of the acetic acid solution. The tube was then immersed in boiling water for 90 sec (control experiments having shown that boiling for 3 min did not increase the yield of hormones significantly) and centrifuged for 15 min at 3000 rev/min. The supernatant was decanted and filtered. Extracts of glands of adult rats were prepared in the same manner but the glands were extracted singly, a total of 2 ml. of the acetic acid solution being used.

Acetone extracts. The glands were either treated with acetone according to the British Pharmacopoeia (1958) or, in some instances, kept in 5–10 ml. of dry acetone in a refrigerator for 24 hr and then extracted with acetic acid as described above. To recover oxytocic activity from acetone, distilled water 0·5 ml. was added and the acetone evaporated at 40° C. The residual fluid was washed twice with ether (Ginsburg & Smith, unpublished). Guinea-pig and human glands were extracted with acetic acid only.

Calculation of body surface. Heller's (1952) formula for rats aged 12–36 hr was used for rats aged up to 7 days, and Lee's (1929) formula for adult rats, for rats aged 14 days and over. An error—which may be considerable—was thereby introduced into the calculation of the surface of rats in all age groups except in 1-day-old animals and adults. For example: if the body surface of a rat aged 14 days and weighing 20 g is calculated according to the formula for adult animals the results would be 75·6 cm^2; if the formula for new-born rats is used the surface would be 47·4 cm^2. The error does, however, decrease if Heller's formula is used for rats younger than 14 days and Lee's formula for older rats.

Calculation of kidney weight. Donaldson's (1915) figures were employed since they were shown (Heller, 1952) to be applicable to the strain of rats used.

Analyses

Water content of tissues. The animals were killed by decapitation and bled. All brain tissue rostral to the tentorial level was removed, stripped of the dura mater and dried at 100° C. Neural lobes were removed with the help of a dissecting microscope, placed on tared pieces of platinum foil, weighed on a micro-torsion balance, placed in small weighing bottles and dried.

Vasopressor assay. Dekanski's (1952) method was used, but dibenyline was injected instead of dibenamine.

Antidiuretic assay. Ginsburg & Heller's (1953) procedure was used, with the modification that the bladders of the assay animals were cannulated and the readings were taken every 4 min.

Oxytocic assay. The method used was that recommended in the British Pharmacopoeia (1958), but oestrous rats were used and the suspension fluid was modified in that the calcium concentration was halved and the magnesium concentration doubled. The temperature of the fluid in the organ bath of 2·5 ml. capacity was slowly raised to 37° C, the uterus having first been subjected to one or two large doses (30–50 m-u.) of Pitocin (Parke, Davis & Co.) at 30° C. These modifications were found to suppress spontaneous contractions of the organ and to make it possible to use 0·2–1·0 m-u. of the hormone as the 'smaller dose' of the standard preparation. In an experiment in which eight groups of 4 doses were randomly applied to the same uterus (Holton, 1948) the limits for R were 0·966 and 1·059; the limit of error for the assay was 4·9%. Assays of extracts of unknown potency were of the standard (2+2) design and four groups of doses were used for each estimation. The milk-ejecting activity of some of the extracts was estimated by the method of Cross & Harris (1952).

Standard preparations. Since the gland extracts contained both neurohypophysial hormones, Pituitrin (pituitary extract; Parke, Davis and Co.) was used for pressor and oxytocic assays in preference to the fractional preparations. Each batch of Pituitrin was standardized against an extract of the international standard powder. Pitressin (Parke, Davis and Co.) was used for antidiuretic assays for reasons which have been discussed by Ginsburg (1956). Synthetic oxytocin (Syntocinon, Sandoz) was used as reference substance in chromatograms.

Inactivation of neurohypophysial hormones with sodium thioglycollate. Vogt's (1953) modification of the procedure of Ames & van Dyke (1951) was used.

Chromatography. Extracts of the glands of infant and adult rats were chromatographed on paper and stained or eluted as described by Heller & Lederis (1958).

Statistical treatment. Where appropriate, results are given as weighted means and their standard errors. The standard deviation of a weighted mean (σ_W) was calculated as follows: $\sigma_W = \sqrt{\left(\frac{S\Delta^2 N}{N-n}\right)}$, where $S\Delta^2$ = sum of squares of deviations from mean, N = number of pooled glands and n = number of assays. Student's t test for small samples as described by Fisher (1944) was used for estimating the significance of difference of means. The probability P for t was obtained from Fisher & Yates's (1943) tables.

RESULTS

Pressor activity in the neurohypophysis of infant rats

Table 1 shows the mean vasopressin content per neural lobe during the first month of extra-uterine life. The figures shown are based on pressor assays; some antidiuretic assays gave similar results. The two methods of extraction used (see Methods) yielded estimates of mean pressor activity which did not differ significantly for each age group.

In order to test whether the neurohypophyses of new-born and infant rats contained not only absolutely but also relatively less activity, the mean glandular hormone content at each age was related to three parameters of comparison, namely, to body weight, body surface and kidney weight. Figure 1 shows that—irrespective of the criterion used—the relative hormone levels were lower for some weeks after birth than those in adults. Pressor and antidiuretic activity per unit weight of wet neurohypophysial tissue was also low in infant animals. For instance, the neurohypophyses of rats aged 7 days (the earliest post-natal date at which the posterior lobe could be reliably dissected) contained a mean of 59 ± 7·5 m-u. pressor activity per milligram, as compared with 564 ± 85·5 m-u./mg for adult neurohypophysial tissue.

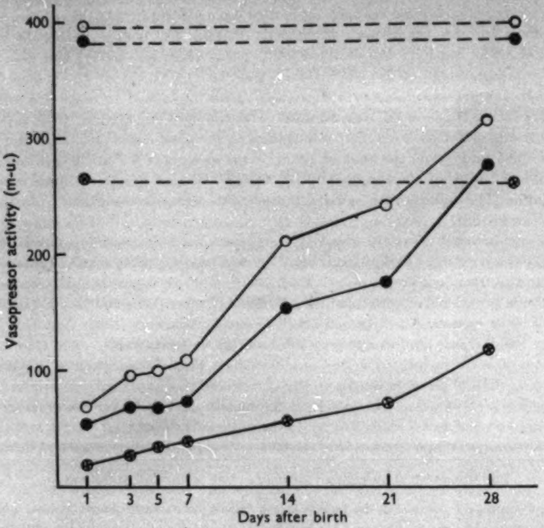

Fig. 1. Mean hormone content (vasopressor activity) in the neurohypophysis of infant and adult rats as related to three parameters of comparison: ○—○, m-u./100 g body weight; ●—●, m-u./g kidney weight; ⊗—⊗, m-u./100 cm² body surface. The horizontal lines indicate mean levels in adults.

Pressor activity in the neurohypophyses of new-born guinea-pigs and human infants

The hormone content of the neural lobe of infant and adult guinea-pigs was estimated, to obtain results in a species which is much more developed at birth than the rat. The large amounts of vasopressor activity found in the pituitaries of new-born guinea-pigs (Table 2) are in keeping with this difference in maturity. Human posterior lobes were investigated because it seemed advisable to repeat the observations of Heller & Zaimis (1949) with more recent methods of assay. The results (Table 2) were in good agreement with the older findings. In spite of great variations within each age group, mean hormone content per milligram of gland was again distinctly lower than in adults, but there was no difference when hormone content was referred to body weight.

Table 1. Vasopressor and oxytocic activity in the neurohypophysis of infant and adult rats. For details of the two methods of extraction used see text (p. 300)

Post-natal age (days)	Acetic acid extracts				Acetone extracts			
	Mean body weight (g)	Number of glands	Vasopressor activity* (m-u./gland)	Oxytocic activity* (m-u./gland)	Mean body weight (g)	Number of glands	Vasopressor activity* (m-u./gland)	Oxytocic activity* (m-u./gland)
1	5·9	46	3·7 ± 0·2	3·3 ± 0·8	6·5	67	4·6 ± 0·5	0·6 ± 0·1
5	10·8	52	11·4 ± 0·6	4·3 ± 0·5	11·2	68	11·0 ± 1·0	1·4 ± 0·2
7	14·3	19	13·5 ± 0·6	10·9 ± 2·0	11·6	18	14·6 ± 3·7	3·6 ± 1·5
14	20·5	20	36 ± 8·2	27 ± 3·4	19·8	19	49 ± 5·4	28 ± 4·9
21	29·9	22	71 ± 10·7	55 ± 12·7	37·5	28	91 ± 12·5	52 ± 6·2
28	53·1	17	139 ± 17·6	144 ± 6·3	49·7	16	155 ± 30·1	125 ± 8·8
Adult	216·0	12	854 ± 73·0	683 ± 94·2	211·0	4	839 ± 85·7	732 ± 96·8

* Weighted means ± s.e.

Table 2. Hormone content (vasopressor activity) in the neurohypophysis of infants and adults of different species. Means ± s.e.

Species	Post-natal age (days)	Infants				Adults			
		Number of glands	(m-u./gland)	(m-u./mg wet gland)	(m-u./100 g body wt.)	Number of glands	(m-u./gland)	(m-u./mg wet gland)	(m-u./100 g body wt.)
Guinea-pig	1	5	398 ± 120	412 ± 65	496 ± 181	4	2,325 ± 546	639 ± 123	513 ± 114
Man	Up to 3	6	460 ± 107	32 ± 5·7	24 ± 4·4	5	15,230 ± 2,610	125 ± 42	23 ± 10·0
Rat	7	35	13·5* ± 1·7	59* ± 7·5	117* ± 49·5	16	850 ± 57	564 ± 86	400 ± 30

* Weighted means.

It is impossible to say whether the variations within age groups were 'genuine', due to terminal hormone release or hormone inactivation after death, or to a combination of these causes.

Oxytocic activity in the neurohypophysis of infant rats

While at all ages investigated much the same means for pressor activity per gland were obtained, irrespective of the method of extraction employed, considerably less oxytocic than pressor activity was found when the pituitaries of very young rats were treated with acetone before extraction with acetic acid (Table 1). The vasopressor:oxytocic ratios (V:O) in acetone-treated glands of animals of less than 14 days of age were therefore much higher than those in glands of the same age groups which had been extracted with acetic acid only (Fig. 2).

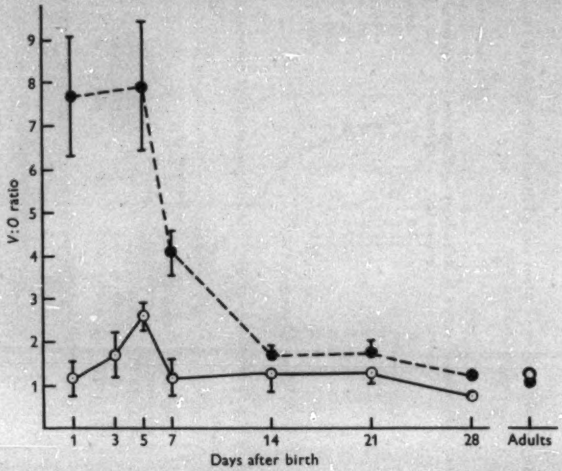

Fig. 2. The relation of vasopressor (V) to oxytocic (O) activity in extracts of infant and adult rat pituitaries as dependent on the method of extraction. ●---●, glands treated with acetone and subsequently extracted with dilute acetic acid (see Methods, p. 300); ○—○, glands extracted with acetic acid only: means and s.e. The numbers of glands in each age group were the same as in Table 1.

These results raised the question whether the glands of infant rats contain an oxytocic substance, other than oxytocin, which is soluble in acetone. In favour of this possibility was the observation that frequently the isolated rat uterus, when in contact with extracts of pituitaries of rats aged less than 7 days, relaxed less readily than when exposed to extracts of the glands of older animals or to Syntocinon. Against it are the following findings: (*a*) The oxytocic activity in 'acetic acid' extracts of infant pituitaries was completely inactivated by thioglycollate (8 experiments). The oxytocic activity in the acetone with which infant glands had been treated was likewise inactivated by thioglycollate (4 experiments). (*b*) When acetic acid extracts of infant rat pituitaries were chromatographed on paper according to the procedure of Heller & Lederis (1958) the proportion of oxytocic activity which could be eluted at the R_F of synthetic oxytocin was the same as that eluted from chromatograms of adult rat glands (Fig. 3). (*c*) The milk-ejecting potency of 'acetic acid extracts' of infant rat pituitaries compared satisfactorily with their oxytocic activity (3 experiments).

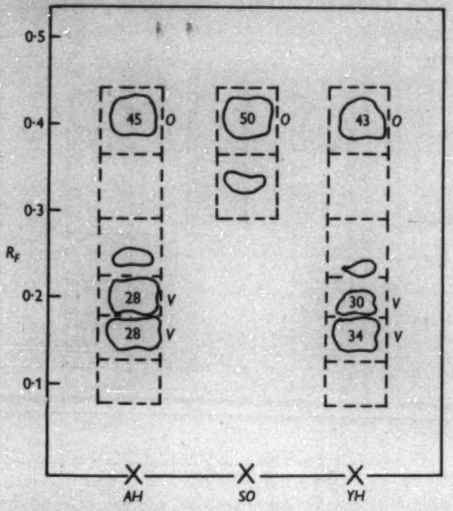

Fig. 3. Ascending paper chromatograms of acetic acid extracts of neurohypophyses of adult rats (*AH*) and of rats aged 7 days (*YH*); and synthetic oxytocin (*SO*). Two identical chromatograms were prepared but only one was stained. The other was cut into pieces indicated by the broken lines. The pieces were extracted, and oxytocic (*O*) and vasopressor (*V*) activities in the eluates were assayed. The figures within the 'spots' indicate percentage recovery of activities applied at the origin (X).

These findings make it unlikely that more than traces of an oxytocic principle other than oxytocin occur in the gland of infant rats and they suggest that acetone extracts oxytocin preferentially to vasopressin. To investigate the matter further, the following experiments were done on neural lobes of adult animals. Ox posterior pituitary glands, obtained immediately after killing, were treated with acetone according to the pharmacopoeial procedure

(British Pharmacopoeia, 1958), i.e. the glands were placed in acetone (4 ml. per gland), removed after 3 hr and placed in a similar volume of acetone where they remained during the night. The two portions of acetone were pooled, diluted with distilled water and the acetone removed. A part of this residual fluid was taken up in Ringer–Locke solution for oxytocic and pressor assay, another part was used for paper chromatography. All such extracts (4 experiments) contained oxytocic activity which on chromatograms moved at the same rate as synthetic oxytocin (Fig. 4) and which was completely inactivated by thioglycollate. The amounts of oxytocin extracted by acetone from these ox glands were small (Table 3), in fact so small that the $V:O$ ratios were not much affected. No vasopressor activity could be demonstrated in the acetone residue.

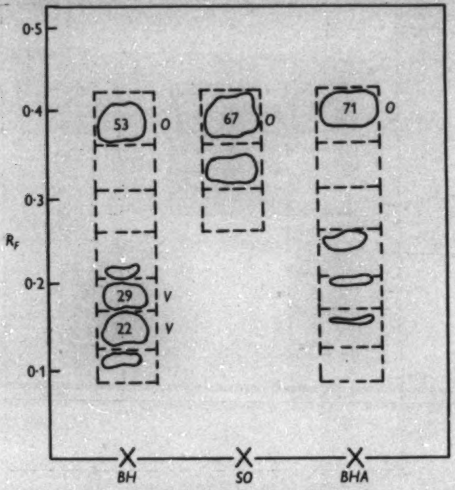

Fig. 4. Ascending paper chromatograms of an acetic acid extract of an ox neurohypophysis previously treated with acetone (*BH*), of the oxytocic activity in the acetone with which the bovine gland had been treated (*BHA*) and of synthetic oxytocin (*SO*). The figures within the 'spots' indicate percentage recovery of activities applied at the origin (X). *O*, oxytocic activity; *V*, vasopressor activity. No activity could be found in the eluates of spots for which no figures are given. For further details see legend to Fig. 3.

Experiments with extracts of neurohypophyses of adult rats gave similar results; i.e. only about 2% of the total oxytocic activity could be recovered from acetone. However, when the glands of infant rats were subjected to the same procedures this percentage rose to about 60 in the case of 5-day-old animals (Table 3).

TABLE 3. Oxytocic activity recovered from acetone with which pituitary glands had been extracted

Post-natal age (days)	Oxytocic activity in acetone* (m-u./gland)	Activity extracted with acetic acid after treatment with acetone		$V:O$	$V:O$† corrected	Percentage of total oxytocin content recovered from acetone
		Oxytocic activity (O) (m-u./gland)	Vasopressor activity (V) (m-u./gland)			
		Ox glands				
Adult	2370	78,900	89,200	1·13	1·09	2·9
Adult	1700	46,800	68,500	1·46	1·41	3·6
Adult	1320	73,100	83,400	1·19	1·12	1·8
Adult	1820	50,800	65,800	1·28	1·24	3·6
		Rat glands				
5	2·7	1·5	9·5	6·3	2·5	59·2
10	2·4	6·9	—	—	—	34·8
28	14·0	130·0	200·0	1·5	1·4	10·8
Adult	10·7	555·0	—	—	—	1·9

* No vasopressor activity could be found in the acetone. † The oxytocic activity recovered from acetone was added to that extracted with acetic acid and the sum assumed to be the total activity initially present in the gland. This assumption introduces an error, since recovery from acetone was not complete.

The water content of the acetone in which the posterior pituitary glands are placed seems to be one of the factors which determine the amount of oxytocin which is extracted. This is suggested by the results of the following experiments: A number of neurohypophyses of adult male rats were dried over phosphorus pentoxide, powdered, mixed and the powder divided into several portions which were weighed to 0·1 mg. One portion was extracted with acetic acid only, another was first treated with analytically pure acetone (which—according to the British Drug Houses Laboratory Chemicals Catalogue, 1958—may contain up to 0·7% (v/v) water) and a third with acetone to which 1% (v/v) of distilled water had been added. The mean $V:O$ ratio in three experiments with 8, 20 and 22 glands was 1·2 when the powder had been extracted with acetic acid only; the mean $V:O$ ratio after extraction with 'dry' acetone was 1·6 and that obtained after treatment with watery acetone was 2·1.

In several experiments with rat pituitaries the weight of wet neurohypophysial tissue per unit volume of acetone used for extraction (6·5 ml./mg) was the same for adult and infant glands, but in view of the foregoing results it seemed advisable to determine their water content. The brains of infant rats were found to contain considerably more water than those of adult animals: the brain of 1-day-old rats contained 88·0 ± 0·15% (6) water, that of 8-day-old rats 88·0 ± 0·10% (6) and that of adult rats 78·6 ± 0·20% (6). However, the water content of the neural lobe of 8-day-old rats was 74·6 ± 1·30% (7) as against 75·5 ± 1·24% (8) in adult rats: that is to say it was much the same ($P > 0·6$). Similarly, small differences in the water content of the posterior pituitaries of human infants and adults had already been noted by Heller & Zaimis (1949).

Effect of dehydration on the hormone content of the neurohypophysis of infant rats

Figures 1 and 2 show that, according to the parameter of comparison chosen, the pituitaries of new-born rats contained 6–14 times less pressor and oxytocic activity than those of adult controls. Morphological evidence—which will be discussed later—suggests that this is due to immaturity of the hypothalamo-neurohypophysial system in this species at birth and for some time after. It may therefore be asked whether, at this stage of development, this complex neuro-endocrine system can be made to respond to a 'physiological' stimulus like dehydration which is known to increase the secretion of vasopressin in adults. When infant rats were separated from the mother for 24 hr (but were protected from cold) it could be shown that their neural lobe contained less pressor activity than that of litter-mates which had remained with her. The mean decrease in hormone content in dehydrated (and starving) rats aged 1–2 days was 27·1% (17), that in rats aged 4–5 days 23·3% (5) and that in rats aged 7–8 days 20·6% (6). Vasopressor–antidiuretic activity in the neural lobe of male adult rats deprived of water and food for 24 hr was 1332 ± 135 (6) m-u. as compared with 1036 ± 135 (6) m-u. per gland in controls with free access to food and water. The difference is not significant, but it becomes so when hormone contents per 100 g body weight are compared (dehydrated rats: 604 ± 67 m-u./100 g, controls 408 ± 64 m-u./100 g; $P = 0{\cdot}05$). A slight rise in hormone content after 24 hr seems likely, considering that a pronounced increase in hormone storage has been shown to occur in rats when drinking water is withdrawn for 48–72 hr (Ames & van Dyke, 1950; Dicker & Nunn, 1957). It seems that adult rats have to be dehydrated for over 96 hr before the hormone content begins to fall (Simon, 1934; Dicker & Nunn, 1957).

DISCUSSION

In the main the maturation of the hypothalamo-neurohypophysial system has been judged by three criteria: (*a*) the differentiation and development of the cell bodies of the nuclear neurones, (*b*) the abundance and microscopical appearance of neurosecretory material, and (*c*) the hormone content of the neural lobe. Concerning the first of these, it has been shown by Auer (1951) that in the golden hamster the hypothalamic nuclei appear as separate structures only after birth. The last nuclear mass to differentiate is the supraoptic nucleus, which is still difficult to identify on the fifth post-natal day. In the dog and in man, on the other hand, the hypothalamic nuclei are clearly discernible before birth (Papez, 1940). Cytological features of post-natal nuclear cell maturation have been described by Rodeck (1958) in the rat and other mammalian species including man.

The results of the numerous investigations on the first appearances of gomori-positive material in the hypothalamo-neurohypophysial system (Bargmann, 1949; Dawson, 1953; Benirschke & McKay, 1953; Scharrer, 1954; Diepen, Engelhardt & Smith-Agreda, 1954; Green & van Breemen, 1955; Rodeck & Caesar, 1956; Rodeck, 1958; Amoroso, Harrison, Harrison-Matthews, Rowlands, Bourne & Sloper, 1958) can be summarized as follows. At birth the neurosecretory material is scanty in those forms which are also immature by other criteria. Usually it appears first in the neural lobe, but in the rat even there only on the 4th to 6th post-natal day in the form of very fine dust-like granules which aggregate as the animals get older. It can therefore not be excluded that the hormones may occur in the hypothalamo-neurohypophysial system—perhaps at some developmental stages only— in a form which cannot be demonstrated microscopically or ultramicroscopically. This would limit the value of the histological approach considerably. It seems desirable, therefore, to relate microscopical findings to results of hormone estimations by biological assay. This has to some degree been attempted in the present investigation. Small amounts of pressor and oxytocic activity were found in the neurohypophyses of rats killed during the first days after birth, that is at a stage of development when chrome-alum-haematoxyphile material cannot be demonstrated under the light microscope. Electron-microscopical investigations—now in progress—may show in what form the active material is stored in the pituitary at this stage of development.

The neurohypophyses of new-born guinea-pigs and human beings were considerably richer in hormone than those of rats of the same age. If, for example, vasopressor activity per milligram of gland is compared and the ratio adult: infant is calculated from the results shown on Table 2, the ratio for 7-day-old rats is 9·5, that for human infants aged up to 3 days is 3·9 and that for guinea-pigs aged 1 day 1·6. There appears thus to be a satisfactory parallelism between the ontogeny of the hypothalamo-neurohypophysial system, as gauged by pituitary hormone content and abundance of neurosecretory material, and over-all maturity at birth as judged by such criteria as the eruption of teeth, the development of locomotion and the opening of the eyes (Koldovský, Křeček, Křečková & Mikulaš, 1953). If these correlations apply to mammals generally, it can be expected, for instance, that the neurohypophysial hormone content in the new-born hamster (Auer, 1951) resembles that of new-born rats, but that the hormone levels in new-born seals (Amoroso *et al.* 1958) are more similar to those in new-born guinea-pigs.

Hormone assays unsupported by histological evidence cannot reveal whether the low amounts of active principles in an endocrine tissue are due to increased release or to slow rate of synthesis. But, in conjunction with the morphological evidence already discussed, it seems likely that the neurohypophysis of the new-born rat contains less hormone either because the rate of production is low or because the submicroscopical structures concerned in hormone storage (Palay, 1957; Bargmann, 1958) are as yet incompletely developed. There is also the possibility that the hormones found in the neurohypophysis of immature animals are peptides which differ chemically from the adult hormones and which are less potent. However, since it could be shown in the present paper (Fig. 3) that the active principles extracted from the neural lobe of infant rats behave chromatographically like arginine-vasopressin and oxytocin, this is unlikely. Whether the infant rat gland contains in addition traces of substances not present in adult neural lobes which may affect the isolated uterus or the rat's blood pressure needs further study.

Recent investigators (Dicker & Tyler, 1953; Acher *et al.* 1956) interested in the hormone content of immature posterior pituitary glands have reported that the ratio of vasopressor to oxytocic activity ($V:O$ ratio) in the rat pituitary is very high shortly after birth, reaching approximate unity in the third post-natal week. The high ratios in very young animals have been confirmed, but only when—as recommended by the British Pharmacopoeia (1958)—the glands were treated with acetone before extracting with dilute acetic acid.

When the acetone treatment was omitted the mean $V:O$ ratios in the glands of rats aged 1–5 days varied irregularly from 1·2 to 2·7 and were near unity in older animals. That oxytocin is preferentially extracted by acetone is not surprising, since it is known (Acher & Fromageot, 1955) to be generally more soluble in organic solvents. It is, in fact, owing to this property that the two peptides can be separated by counter-current distribution. The higher solubility of oxytocin could also have been deduced from the work of Dudley (1923), who at one stage of his attempts to separate the neurohypophysial hormones obtained a powder of high oxytocic potency a twentieth of which could be extracted with 'dry' acetone. Similar results were obtained by us when ox or adult rat pituitaries were extracted. Moreover, it could be shown that the oxytocic material extracted by acetone was inactivated by thioglycollate and that on paper chromatograms it moved at the same rate as synthetic oxytocin.

We cannot say at present why relatively more oxytocin can be removed by acetone from infant than from adult rat glands, but this is apparently not the only physiological situation in which this phenomenon occurs. It can also be shown in the neural lobes of lactating rats in which again $V:O$ ratios are high when acetone is used (Dicker & Tyler, 1953; Acher *et al.* 1956), but in which the ratios do not differ significantly from those in non-lactating controls when simple acetic acid extraction is used (Rennels, 1958; Heller & Lederis, 1959). It remains to be seen whether these disparities in solubility are due to modifications of the peptide-carrier complex or whether perhaps they are produced by cytological differences in storage.. The difference between the reactions to stains of neurosecretory material in the pituitaries of infant and adult rats (Dawson, 1953) may be worth noting in this connexion.

While the amount of hormones stored in the neural lobe of infant rats was very low as compared with adults, it seems possible that the mechanism for release of the vasopressor-antidiuretic factor by dehydration operates already shortly after birth. Our finding, that the pituitaries of infant rats deprived of milk for 24 hr contained 20–30 % less vasopressor-antidiuretic activity than those of litter-mate controls, could be so interpreted. It would be remarkable if this applied, for it would mean that antidiuretic hormone is released at an age when the kidneys are unable to respond to it (Heller, 1949; Adolph, 1957). The alternative explanation could be that the neuro-hormonal reflex is not activated, but that withdrawal of food and fluid at so early an age inhibits hormone synthesis or transport. In adult rats withdrawal of food and water for the same period stimulates both the release and—as is shown by the concomitant increase in hormone storage—the rate of synthesis of the antidiuretic principle.

SUMMARY

1. Vasopressor and oxytocic activities were estimated in the pituitary glands of rats from the post-natal age of 1 to 28 days. When compared with the neurohypophyses of adults, the pituitaries of infant rats contained substantially less vasopressor and oxytocic activity. This applied whether hormone content was expressed per milligram of gland or whether it was related to body weight, body surface or kidney weight.

2. The neural lobe of new-born guinea-pigs was considerably richer in activity than that of new-born rats. The hormone content of the neurohypophysis of human infants aged up to 3 days was intermediate.

3. The ratio vasopressor:oxytocic activity in the glands of rats aged 1–5 days varied irregularly from 1·2 to 2·7 and was near unity in older animals.

4. Watery acetone was shown to extract small amounts of oxytocic activity from ox and adult rat neurohypophyses. When chromatographed on paper the oxytocic material extracted was found at the same R_F as that of synthetic oxytocin and was inactivated by thioglycollate. No pressor activity could be detected in the acetone.

5. Acetone extracted relatively larger amounts of oxytocic material from the pituitaries of rats aged up to about 7 days than from the glands of adult animals.

6. After withdrawal of milk for 24 hr the neurohypophyses of rats aged 1–8 days contained less vasopressor activity than the glands of litter-mate controls. In contrast, the neural lobes of adult rats which had been deprived of food and water for the same period contained slightly higher amounts of activity than those of animals with free access to food and water.

7. The relation of these results to morphological findings in the neurosecretory system of infant animals and to over-all maturity at birth is discussed.

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THE H POTENTIAL AND THE CONDUCTION VELOCITY OF THE BUNDLE OF HIS

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The conduction velocity of the bundle of His has been estimated so far only by indirect means. The availability of a method for recording the activation of the bundle at various regions (Alanís, González & López, 1958) made it possible to measure this velocity directly. The purpose of the present study was to determine the conduction velocity and the shape of the action potential of the bundle of His and their modifications during various experimental conditions.

METHODS

The experiments were carried out on isolated perfused hearts of dogs, calves and goats with the technique previously described (Alanís *et al.* 1958). The right auricle or the right ventricle was stimulated with rectangular pulses. The recording electrodes were either steel needles, 4–5 mm long, with their tips sharpened to a diameter of 5–30μ, or glass micropipettes filled with KCl solution and having a diameter of 4–16μ at the tip, made according to the technique of Ling & Gerard (1949) as modified by Hoffman & de Paes (1958). The action potentials were amplified by a three-stage push-pull resistance-capacity-coupled amplifier in some cases, and in others by a DC push-pull pre-amplifier after passing them through a cathode follower. These potentials were recorded with a double-beam cathode-ray oscilloscope. The monophasic action potentials were recorded from an intact to an injured region of the bundle of His.

The conduction velocity was determined by measuring the difference of latency between two points. For this purpose in some cases two pairs of electrodes were placed on different segments of the bundle of His. In others, only two electrodes were employed, each recording against ground. In most experiments the bundle of His was blocked by severing it below the region where the recording electrodes were placed. The heart was subjected to periods of asphyxia by the occlusion of the aortic cannula.

Acetylcholine (Roche Products) and adrenaline (Colliere) were injected directly into the aorta of the receptor heart. The vagus nerves were stimulated at frequencies that varied between 20 and 30 per second.

RESULTS

The monophasic electrogram of the bundle of His

When the bundle of His was activated through the auricle, an action potential (H potential) was obtained which was similar in every respect in the different species studied. The H potential recorded from an intact to an injured region was monophasic, with two components; an initial rapid depolarization followed by a slow repolarization phase (Fig. 1). The amplitude of the H potential varied from 6 to 18 mv. In the experiments in which the bundle was not severed and the impulses reached the ventricle, the ventricular response developed during the later part of the slow component of the H potential (Fig. 1). The average duration of the monophasic electrogram, measured from the initiation of the rapid depolarization phase to the end of the repolarization period was, 200 msec.

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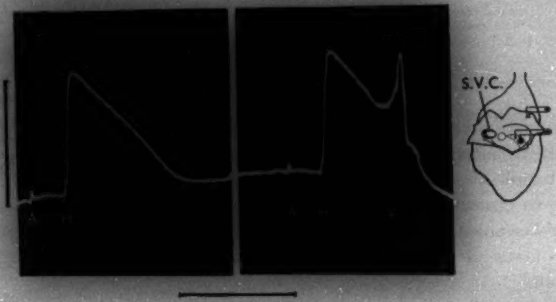

Fig. 1. Monophasic potentials of the bundle of His. Perfused dog heart; bundle of His stimulated at constant frequency through the auricle. The diagram shows the position of the stimulating and recording electrodes. Records were derived from an intact to an injured region and passed through a DC pre-amplifier. A, auricular electrogram; H, potential from the bundle of His; V ventricular potential. The first potential was obtained from a heart in which the bundle of His was severed below the region where the recording electrodes were placed; the second record was obtained before the severance of the bundle of His. Calibrations, 100 msec and 12 mV.

Factors which modified the H potential

When the bundle of His was activated through the auricle at progressively increasing rates, the H potential was modified in its shape as well as in its duration. These changes occurred mainly at the expense of the slow phase of repolarization, which was shortened. The progressive shortening of the potential is illustrated in the curve of Fig. 2. The maximal shortening was attained at the highest rate of stimulation and reached 72% of the initial value, on the average. Stimulation of the vagus nerves, injections of acetylcholine and adrenaline, and periods of asphyxia also shortened the slow phase of the H potential.

The conduction velocity

The conduction velocity of the impulses varied considerably. When the impulses were conducted from auricle to ventricle (orthodromically), the velocity was on the average slightly greater than when the impulses travelled in the opposite direction (antidromically). Both the orthodromic and antidromic condition velocities were determined without any change in the position of the recording electrodes and at the same rate of stimulation, which was kept constant.

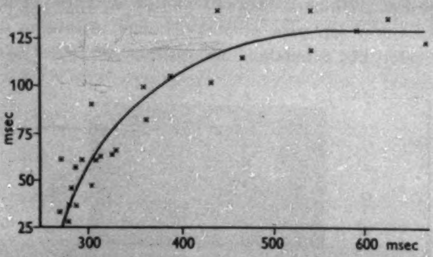

Fig. 2. Changes in duration of H potential produced by increasing the frequency of stimulation to the auricle. Abscissae, interval between stimuli, ordinates, duration of the potentials, measured from the beginning to 0·33 of the maximal amplitude on the descending branch.

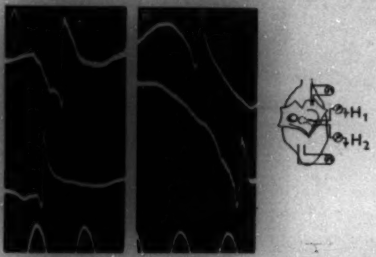

Fig. 3. Differences in the activation time of two regions of the bundle of His. The diagram shows the position of the stimulating and recording electrodes. The H_1 potential (lower tracings) corresponds to records obtained from the electrode H_1, near the anterior edge of the coronary sinus. The upper tracings correspond to the activation of the region stiuated beneath the H_2 electrode. A, auricle stimulated at a constant rate, 1·2/sec; B, records taken from the same electrodes when the ventricle was stimulated at the same frequency. This is an atypical experiment which shows a greater orthodromic than antidromic conduction velocity. Time marker, 10 msec.

The conduction velocities were measured from records similar to those shown in Fig. 3. The mean value of 91 measurements for the orthodromic conduction velocity was 1·26 m/sec, s.e. ± 0·04. The mean value of the antidromic conduction velocity obtained from 24 measurements was 1·16 m/sec, s.e. ± 0·08. The difference between the orthodromic and antidromic conduction velocity was not statistically significant.

Changes of conduction velocity

When the bundle of His was activated orthodromically at increasing rates, the conduction velocity diminished progressively. The maximal reduction obtained by this procedure was 62·8% of the initial value, on the average, and was attained at the highest rate of stimulation. These changes are shown in Fig. 4. Periods of asphyxia or acetylcholine injections diminished the conduction velocity, but adrenaline increased it.

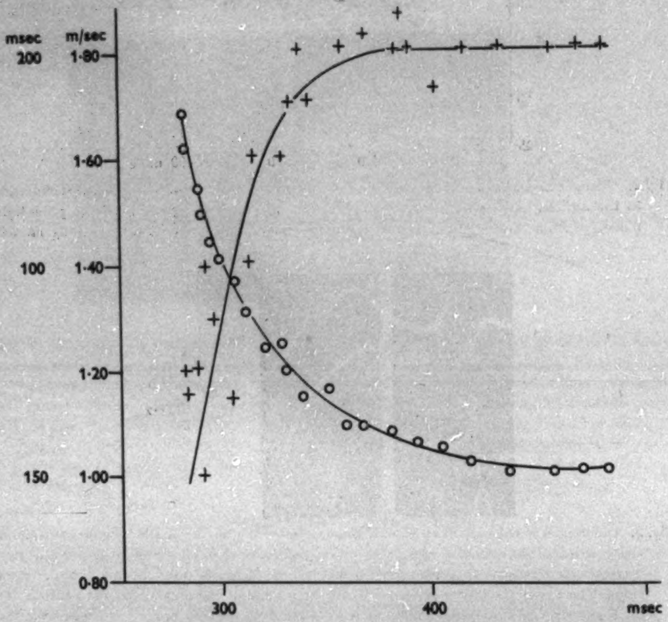

Fig. 4. Changes of the duration of the A–H subinterval (○) and of the conduction velocity (+), produced by an increase of the frequency of stimulation to the auricle. Abscissae, time interval between stimuli; ordinates, left scale, magnitude of the A–H subinterval; right scale, conduction velocity. Note that the maximal percentage lengthening of the A–H subinterval is greater than the maximal percentage reduction of the conduction velocity.

The V–H and H–A subintervals

When the heart was driven through the ventricle, each impulse reached the auricle with a certain delay (V–A interval). The V–A interval was divided into two subintervals by the H potential, namely, the V–H and H–A subintervals

(Fig. 5). The mean value of the V–H subinterval was 28 msec. The H–A subinterval had a mean value of 94 msec and represents 77 % of the V–A interval.

The effect of frequency of activation on the V–H and H–A subintervals

The progressive increase in the frequency of ventricular stimulation lengthened the V–H and H–A subintervals (Fig. 6). The H–A subinterval increased gradually and reached its maximal value when the impulse failed to activate the auricle at an average frequency of 2·7/sec. The duration of the V–H subinterval did not change until frequencies above 4·3/sec were reached. The maximal lengthening of the V–H subinterval coincided with the highest frequency at which the bundle of His could be activated through the ventricle (Fig. 6).

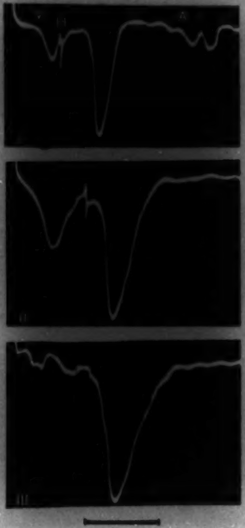

Fig. 5. Lengthenings of the V–H subinterval and disappearance of the H potential produced by progressive increase of the frequency of stimulation of the ventricle. I, stimulation frequency 1·5/sec; II, frequency 2·8/sec—note the disappearance of the auricular potential and the lengthening of the V–H subinterval; III, frequency 5·5/sec—the H potential disappears while the ventricular electrogram is still present. Time marker, 100 msec.

Maximal frequency of activation of the bundle of His through the auricle

In order to determine the refractory period for the propagation of impulses from the auricle to the bundle of His, two procedures were employed. In some instances the bundle was stimulated through the auricle, driven at gradually increasing frequencies. The H potential disappeared when the frequency of stimulation was greater than 4·5/sec on the average. In other observations the functional refractory period of the propagation from the auricle to the bundle of His was measured. The functional refractory period has been defined as the minimal interval between two propagated responses (Rosenblueth, Alanís & Mandoki, 1949; Krayer, Mandoki & Méndez, 1951; Rosenblueth, 1958). At the frequency of stimulation used, the functional refractory period had a mean value of 318 msec. The maximal frequency which the auricle followed was 7·5/sec, on the average, and its functional refractory period determined by pairs of stimuli had a mean value of 231 msec at a frequency of 1·2/sec.

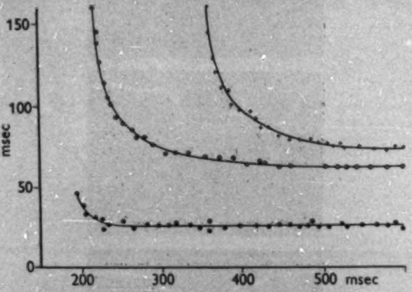

Fig. 6. Changes of the duration of the H–A subinterval (+), and of the V–H subinterval (●), when the frequency of stimulation to the ventricle was progressively increased. ○, changes of the A–H subinterval provoked by increasing rate of stimulation to the auricle of the same heart. Abscissae, interval between stimuli; ordinates, magnitude of the subintervals. Note that the lengthenings of these subintervals occurred at different frequencies of stimulation.

Maximal frequency of activation of the bundle of His through the ventricle

When the ventricle was driven with stimuli at progressively increasing rates, the maximal frequency followed by the bundle of His was 5·6/sec on the average (Fig. 6); the ventricle followed higher frequencies (6·5/sec) without fibrillating (Fig. 5).

DISCUSSION

Relationship between the potential of the bundle of His and the ventricular activation

By different experimental procedures it was possible to shorten the duration of the H potential. The average magnitude of this shortening was 75%, without interfering with the activation of the Purkinje tissue and ventricular muscle. The activation of the bundle of His at progressively increasing frequencies through the auricle does not change the latency between the H potential and the ventricular electrogram (H–V subinterval; Alanís *et al.* 1958), but the H potential is shortened considerably. These facts suggest that under the present experimental conditions the important factor for the activation of the bundle of His and the Purkinje fibres is the magnitude of the depolarization rather than the duration of the H potential. Similar findings have been reported by Burgen & Terroux (1953) for the A–V propagation.

The conduction velocity of the bundle of His

The conduction velocities attributed to the auriculo-ventricular conducting system have varied considerably. Sher (1955) attributed to the bundle of His and the preterminal segment of Purkinje tissue a conduction velocity of 2 m/sec. This figure was obtained by measuring the time necessary to conduct an impulse from the bundle of His to the papillary muscle and dividing it by the interelectrode distance. The complexity of the system studied by this author, comprising the bundle of His, Purkinje tissue and papillary muscle, and the uncertainty of the pathway followed by the impulse, make this method inadequate. The dispersion of the values obtained with the present method may be explained by the following factors.

(*a*) The trajectory of the bundle of His might not be rectilinear. In such a case the interelectrode distance would not correspond to the length of the pathway travelled by the impulse. However, this factor, if present, would be negligible, since the anatomical descriptions of the bundle of His (Testut, 1924; Lev, Widraw & Erickson, 1951) and our physiological mapping (Alanís *et al.* 1958) indicate that the trajectory is approximately rectilinear and does not vary appreciably from one animal to another.

(*b*) In some experiments the shape of the H potential recorded varied from one region to another. This factor made it difficult to measure precisely the difference of latency between the two potentials. To reduce this source of error, measurements of the conduction velocity were carried out only in those experiments where all potentials were similar in shape and had precise points of reference (Fig. 3).

(*c*) The estimation of the interelectrode distance is subject to some error due to movement of the heart. This was probably the most important source of error and could not be overcome.

Changes in the conduction velocity

In the bundle of His the conduction of impulses travelling during the relatively refractory period is slower than in completely recovered tissue. This observation agrees with the findings of Lewis (1925) in other cardiac muscles. It was reported previously (Alanís *et al.* 1958) that an increase in frequency of stimulation, asphyxia or vagal stimulation, all lengthen the auricle–His latency (A–H subinterval). It was stated that this lengthening takes place above the bundle of His, i.e. in the region of the A–V node. Our present results confirm this assertion since the percentage reduction in the orthodromic conduction velocity of the bundle of His was smaller than the percentage increase of the A–H subinterval (Fig. 4).

The H–A subinterval

The time taken by an impulse to travel from the bundle of His to the auricle (H–A subinterval) is 94 msec, while the total time required by an impulse to propagate from the ventricular muscle to the auricle (V–A interval) is 132 msec. The length of the bundle of His is approximately 25 mm. The recording electrodes were placed near the middle of this structure; the distance between the recording region and the A–V node was thus 10–12 mm. Using the values obtained for the antidromic conduction velocity of the bundle, the time required for the impulses to travel this distance would be 13·5 msec. This figure represents only 14% of the H–A subinterval, indicating that the major delay occurs in the region of the A–V node. At increasing frequencies, the H–A subinterval lengthened progressively until the propagation to the auricle failed, in spite of the presence of the H potential. This fact suggests that the block occurred in the region of the A–V node.

The refractory period

Since the bundle of His was not stimulated directly, its refractory period could not be determined. When it was activated through the auricle the refractory period was larger than those of the auricle and of the bundle of His, as the bundle followed higher frequencies when activated through the ventricle. It may be concluded that the values obtained correspond to the refractory period of the auricle–His propagation. When the bundle of His is stimulated through the ventricle at increasing frequencies, the refractory period again cannot be attributed unequivocally to the bundle since the block may occur before the impulse reaches this tissue. The fact that the ventricular muscle followed higher frequencies than the bundle of His suggests the existence of a functional discontinuity somewhere in the ventricle–Purkinje–His system (Fig. 5) The progressive increase in the V–H subinterval at frequencies at which the block does not appear supports this suggestion (Fig. 6). The available experimental evidence does not allow us to specify the nature and location of this hypothetical discontinuity.

SUMMARY

1. Hearts from dogs, calves and goats were isolated and perfused. The right auricle was incised to explore the region of the bundle of His.

2. The records were taken from an injured and an intact region. The H

potential appeared as a monophasic electrogram with a rapid depolarization phase followed by a slow component, and its average duration was 200 msec.

3. Increasing the frequency of activation of the bundle of His, vagal stimulation, acetylcholine, adrenaline or asphyxia produced a considerable shortening of the slow component of the H potential. In spite of the magnitude of these reductions (85%), the impulses were propagated along the bundle of His to the ventricular muscle.

4. The conduction velocity of the bundle was measured. The average orthodromic (A–V) conduction velocity was 1·26 m/sec and the antidromic (V–A) conduction velocity was 1·15 m/sec. The difference between these values was not statistically significant.

5. A progressive increase in the frequency of stimulation of the auricle, as well as short periods of asphyxia of the heart, resulted in a decrease of the conduction velocity of the bundle. The magnitude of this decrease is not sufficient to explain the lengthening of the propagation time from the auricle to the bundle.

6. The appearance of the H potential between the ventricular and auricular responses divides the V–A interval into two subintervals: ventricle–His (V–H) and His–auricle (H–A). Increasing the frequency of ventricular stimulation produced a lengthening of both subintervals.

7. When the ventricle was stimulated at high frequencies the H potential disappeared while the ventricular electrogram was still present. A functional discontinuity, localized in the ventricle–Purkinje–His system, is suggested in order to explain the lengthening of the V–H subinterval and the disappearance of the H potential.

8. It is concluded that the critical region for the ventriculo-auricular propagation is not the bundle of His itself, but is located at the A–V node.

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SIMULTANEOUS MEASUREMENTS OF CONTRACTIONS AND INTRACELLULAR POTENTIALS IN ISOLATED RABBIT ATRIA EXPOSED TO ACETYLCHOLINE

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There have been several hypotheses concerning the manner in which vagal stimulation or parasympathomimetic substances may weaken the contractions of cardiac muscle, or produce a negative inotropic effect, as some authors prefer to say. Muskens (1898) examined the effect of vagal stimulation on the frog heart, and concluded that 'the diminishing force of contraction under vagal inhibition may mean that fewer and fewer fibres take part in the contraction, because the decreasing power of conduction prevents more and more fibres from being reached by the excitation waves'. Mines (1914) studied the effects of vagal stimulation on the frog heart, eliminating changes in rate by applying atropine locally to the sinus venosus, and observed that contractions diminished in the absence of any failure in conduction. In his words, 'does the weakened contraction under vagus inhibition mean the contraction of fewer fibres, through failure of the excited state to reach the rest, or does it mean the weakened contraction of all or most of the fibres?' Mines favoured the latter view. He observed also that the diminished contractions were associated with a shortened duration of the action potential.

In mammalian atria there is no evidence of 'decremental conduction' after vagal stimulation or exposure to acetylcholine (ACh). On the contrary, conduction velocity in isolated rabbit atria is increased at a time when contractions are reduced (Vaughan Williams, 1954, 1958). Burgen & Terroux (1953) revived in a modified form the view that the contraction of fewer elements might be responsible for the diminution of contractions in the presence of parasympathomimetic drugs. They made simultaneous measurements of contractions and intracellularly recorded potentials and found that the action potentials were shortened, and that the reduced contractions had earlier peaks. They stated that 'during the progressive inhibition of contraction by carbamylcholine (CCh) there is a definite correlation between the shift in time of peak tension and the height of the tension curve, which suggests that the duration of action potential necessary for excitation may differ for different *myofibrils*, so that with increasing brevity of the action potential the more slowly responding fibrils fail to contract'. 'The effect of ACh or CCh would thus be to eliminate progressively that part of the contraction which is due to the later responding elements.' In the present paper some evidence is reported which is relevant to the above hypotheses.

METHODS

The apparatus has already been described (Vaughan Williams, 1955, 1958, 1959). Rabbit atria were placed horizontally in a bath, the temperature of which was controlled at 31° C to within ±0·1°. Contractions were recorded with an RCA 5734 transducer, and displayed on one beam of a Dumont 322 oscilloscope. Intracellular potentials were displayed on the other beam. External potentials, from which conduction velocity was calculated, were recorded from right and left atria with pairs of small platinum bipolar electrodes, and were displayed on a Cossor 1049 oscilloscope. When the atria were beating spontaneously, the action potential from the right atrium triggered the sweep. The screens of both oscilloscopes were photographed simultaneously with a Grass camera; the time at which each exposure was made was also recorded to within 0·1 sec. When the atria were driven electrically, Ag–AgCl electrodes were placed on the tip of the left atrium. All intracellular records were taken from the endocardial surface of the right atrium, some distance from the natural pace-maker. The Dumont oscilloscope sweep was itself made to produce a pulse at the end of its traverse which set in motion the mechanism for shifting one frame in the camera; this in turn operated a shutter to record the time. Consecutive records could thus be obtained on stationary film, the frames being changed automatically between each heart beat.

RESULTS

It was already known (Mines (1914) in the frog; Burgen & Terroux (1953) in the cat) that the diminution of the contractions of atria during vagal inhibition or during exposure to parasympathomimetic substances was associated with a shortened duration of the action potential. In considering the possibility of a causal link between the two phenomena it was of interest to determine how close was their association. Simultaneous observations of contractions and intracellular potential have been made during thirty-two exposures to various concentrations of ACh in eight pairs of rabbit atria. For comparisons between potential and contraction to be significant, however, it was necessary that the micro-electrode should remain in a single muscle fibre during a control period and thereafter during the inhibition of contractions by ACh and during the recovery period. Only five experiments could be regarded as satisfactory by the criteria discussed in a previous paper (Vaughan Williams, 1959) and the results of these have been plotted in Fig. 1. The ordinate shows the peak size of contractions in milligrams, and the abscissa the time in milliseconds taken for the intracellular action potential to recover from its peak to half-way towards the full diastolic resting potential. The correlation is sufficiently good for a causal connexion between the shortening of the action potential and the diminution of contractions to remain a possibility, or at least not to be ruled out as an impossibility.

Burgen & Terroux (1953) had observed not only that the action potential was shorter when the contractions were smaller in the presence of CCh, but also that the time from the beginning to the peak of the reduced contractions was shorter than in normal contractions. This led them to the hypothesis that some myofibrils required a long action potential in order to be excited, and consequently failed to contract when the action potential was short. It was, therefore, of interest to learn what the duration of the action potential would be in a situation in which contractions were larger than normal, and in which it might be presumed that all or most of the myofibrils were active.

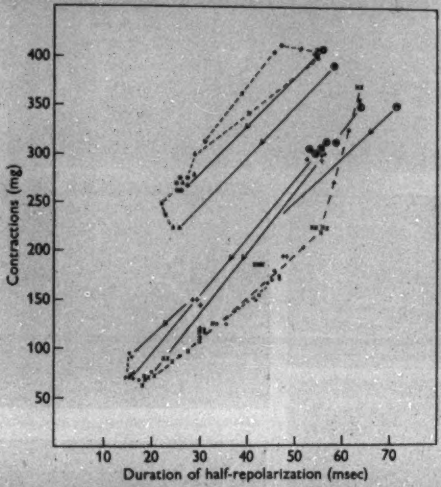

Fig. 1. Correlation between contractions and half-time for repolarization. Different symbols indicate the results of five experiments from four pairs of atria; control observations have been ringed, and the order in which subsequent observations were made has been indicated by arrows.

It is well known that if the atria are arrested by vagal stimulation or by ACh, the first contraction after the pause is larger than normal. This is the reverse of the 'staircase' phenomenon and is illustrated by a smoked-drum record of contractions in Fig. 2*A*. During the phase of inhibition the action potential became very short, the half-time for repolarization being reduced from about 60 msec to as little as 7–10 msec. It was of interest to know whether during the large first contraction the action potential was still short, or had lengthened in proportion to the increase in the contraction. Many attempts were made to record intracellular action potentials and contractions simultaneously in these conditions, but unfortunately the electrode was always pulled out of the fibre by the very large contraction. It was possible, by physically restricting the movement of the atria and so sacrificing the contraction record, to show that the action potential during the first beat after arrest by ACh was short. Fig. 2*B* shows control action potentials before the addition of ACh to the bath. Between *B* and *C* the auricle was stopped for 70 sec by the addition of ACh 5×10^{-5}, and *C* shows the first action potential

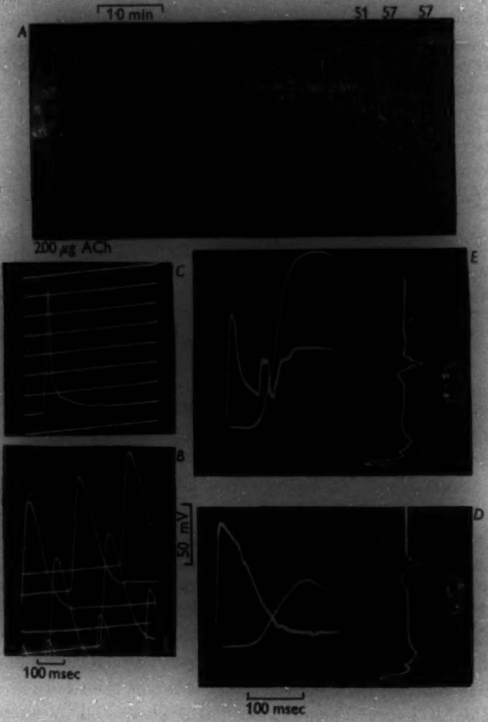

Fig. 2. Arrest of contractions by ACh and recovery. *A*: Smoked drum record to illustrate the large first beat after arrest. *B* and *C*: Records of intracellular potentials taken on moving film before exposure to ACh and after arrest; the first action potential after arrest was short, but there was no contraction record. *D*: Controls; upper trace, intracellular potential; lower trace, contraction; vertical trace on right, action potentials recorded from right and left atria with bipolar surface electrodes, from which conduction velocity could be calculated; inset, time. *E*: First contraction after arrest by ACh for 96 sec. The micro-electrode was pulled out of the fibre by the large contraction, but it was evident that the action potential was short. Note increased conduction velocity.

after the arrest, but in the absence of a contraction record there was only presumptive evidence that the associated contraction would have been larger than normal. Partial success in obtaining simultaneous mechanical and electrical records was eventually achieved on two occasions, illustrated in Fig. 2 *E* and in Fig. 3 *D*. Although there was evidence that the electrode was pulled out of the fibre by the abnormally large contractions and there was some fall of the resting potential, the electrode remained in the fibre long enough to indicate that

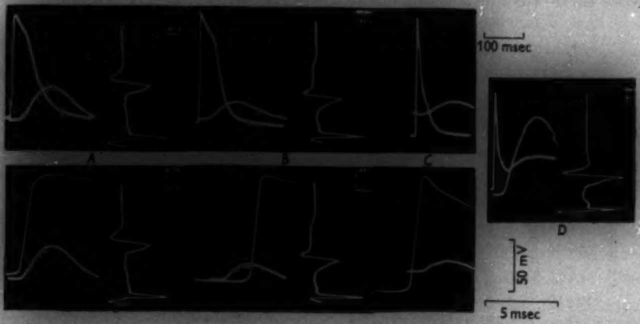

Fig. 3. Records of contractions and intracellular and extracellular potentials during inhibition by ACh and after arrest. *A*: Controls; the upper trace in each frame records the intracellular potential, the lower trace the contraction. The vertical trace records action potentials from bipolar surface electrodes on right and left atria. The dotted lines indicate the zero potential before the micro-electrode was inserted into the fibre. In the lower frames the sweep speed of the trace recording the intracellular potential was much faster, for more accurate measurement of the rate of rise of the action potential. *B* and *C*: Records taken from the same fibre during the action of ACh 2×10^{-5}; the resting potential, the overshoot potential, the rate of rise of the action potential and conduction velocity had all increased. *D*: Record during the first beat after the atria had been arrested for 70 sec.

the action potential was still short. It may also be noted that the peak of the large contraction did not occur earlier than normal. Thus a large contraction in which all or most of the contractile elements were probably active was produced in response to an action potential of very short duration. This would seem to make it less likely that the diminution of contractions by ACh was due to the inability of a substantial proportion of the myofibrils to respond to a short action potential. It might still be maintained on other grounds, of course, that the inhibition of contractions was due to the failure of many contractile elements to be activated, but not on the grounds of an association between a short action potential and an early peak in the reduced contraction, because it is clear that a short action potential can also be associated with a large contraction without an early peak.

It has been suggested (Vane, 1957) that a large part of the inhibition of contractions by ACh may sometimes be attributed to the simultaneous slowing of the rate, as a consequence of the 'staircase' effect. In the present and previous experiments, however (Vaughan Willliams, 1958), it was found that contractions diminished to the same extent whether the frequency of the contractions was allowed to diminish or was kept constant by electrical stimulation. If atria were exposed to ACh in such a way that the drug arrived at the pace-maker last, a large diminution of contractions nevertheless occurred before there was much change in frequency (Fig. 4*A*). If, however, the ACh was directed first on the pace-maker, the atria stopped beating before there had been much diminution in the contractions and when conduction from the right to the left atrium was unaffected (Fig. 4*B*). Webb (1950) stated 'when doses (of ACh) were large, the auricles were stopped when the amplitude fell to zero, the pace-maker in all probability continuing to discharge impulses'. To test this possibility atria were stimulated during the period of arrest by ACh. Even in the presence of concentrations as high as 1 mg/ml., the atria always responded with a contraction. It was concluded, therefore, that the atria were stopped by ACh because of the absence of impulses from the pace-maker, and not as a consequence of a complete inhibition of the contractile mechanism (cf. Hoff, 1955; Hutter & Trautwein, 1956).

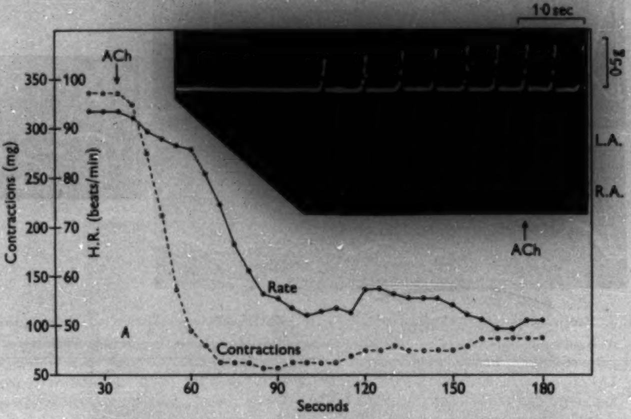

Fig. 4. Independent inhibition of pace-maker and contractions by ACh. *A*: Ordinates, contraction and rate of beat; abscissa, time. At the arrow ACh was introduced to the bath to give a final concentration of 10^{-6}, but the flow was arranged so that it reached the pace-maker last. *B*: Upper horizontal trace, record of contractions on moving film (read from right to left); vertical traces, records of action potentials from right and left atria. At arrow, ACh sufficient to give final concentration of 10^{-5} directed towards pace-maker region; the atria were arrested when contractions were reduced by only 30%.

DISCUSSION

Simultaneous measurements have been made of contractions and intracellular potentials in isolated rabbit atria. It was found that during inhibition of contractions by acetylcholine there was a correlation between the magnitude of contractions and the duration of the action potential (measured as the half-time for repolarization), in agreement with observations made by Burgen & Terroux (1953) of the effect of carbamylcholine on strips of cat atria. These authors had also observed that the small contractions, associated with short action potentials, reached their peaks earlier than normal contractions. They suggested that a substantial proportion of myofibrils required action potentials of long duration in order to become activated, and consequently failed to be excited when the action potentials were short. It was concluded that the short duration of the action potential in the presence of CCh was causally linked to the negative inotropic action of this substance.

It is well known that the first contraction of cardiac muscle after a period of arrest is larger than normal. In the present experiments it was found that when atria were arrested by high concentrations of ACh the first large contraction occurring after the pause was associated with a very short action potential. This contraction, sometimes more than twice the magnitude of control contractions observed before exposure to ACh, had a peak which was not earlier than that of a normal contraction, and was, therefore, presumably produced by activity in all or most of the myofibrils. Thus, although it might still be maintained on other grounds that the diminution of contractions by ACh was due to the excitation of fewer elements, this view was difficult to maintain on the grounds of the association of short action potentials and small contractions with early peaks, since large contractions without early peaks could also be initiated by short action potentials.

If atria were stimulated electrically during the period of arrest by ACh, they always contracted even in the presence of concentrations as high as 1 mg/ml. Further, if atria were exposed to ACh in such a way that the drug reached the pace-maker last, large diminutions in contractions occurred before there was much change in rate. Conversely, exposure of the pace-maker to ACh caused arrest of the atria before there was much diminution of contractions or any effect on conduction from the right to the left atrium.

It was concluded that the *arrest* of rabbit atria by ACh was due to the absence of potentials from the pace-maker and not to the total inhibition of the contractile process, and that the effects of ACh on rate and on contraction were largely independent. Further it would seem that the undoubted association between the diminished contractions and the shortening of the duration of the action potential may be coincidental. The effects of ACh on contractions and on action potentials may both be secondary consequences of a more fundamental action.

SUMMARY

1. Simultaneous measurements of contractions and intracellular action potentials have been made in isolated rabbit atria in the presence of various concentrations of acetylcholine.

2. It was found that the diminution of contractions by ACh was correlated with the reduction in duration of the action potential.

3. If the atria were arrested by ACh, the first contraction after the pause was very large, without an earlier-than-normal peak, but was associated with a short action potential. This makes less probable, as an explanation of the negative inotropic effect, the suggestion that a large number of myofibrils require a long action potential to activate them and therefore fail to be activated by a short action potential.

4. Evidence was presented of the independence of the diminution of contractions and the slowing of the rate by ACh.

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THE METABOLIC RATE OF THE NEW-BORN PIG IN RELATION TO ENVIRONMENTAL TEMPERATURE AND TO AGE

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There is very little information available on the metabolic rate of the new-born pig in relation to its environment. Knowledge of this sort would be of considerable interest, as experience shows that the new-born pig is highly susceptible to chilling, which leads to coma and death, and because the animal has very little hair and subcutaneous fat and may therefore be expected to have a correspondingly low insulation against heat loss. The question arises as to the animal's response to cooling of the environment: whether the new-born pig is poikilothermous like the new-born rat (Fairfield, 1948) and mouse (Fitzgerald, 1953), or whether it responds in a manner typical of the adult homoeotherm, that is by increasing its heat production when the environmental temperature falls below the critical temperature for the animal. The results presented here show that the new-born pig is not poikilothermous and that over the environmental temperature range 4–38° C it exhibits a vigorous metabolic response to cooling of the environment. Further, the critical temperature is not definite, but may be in the region of 34–35° C.

Some of these results have been the subject of preliminary communications (Mount, 1958*a–c*).

METHODS

Observations were made on a total of thirty-six new-born pigs of the Large White breed and eight of the Landrace breed, aged from 1 hr to 7 days and with weights ranging from 0·86 to 2·72 kg. Animals were removed singly from the sow and the rest of the litter in the farrowing pen, and weighed, and the rectal temperature was recorded by a clinical thermometer inserted to a depth of 3·5 cm for 2 min. The pig was then placed, without any other treatment, and without food or water, in the chamber of a closed-circuit metabolism apparatus. The animal was in the dark, and remained quiet for the duration of the experiment; only very rarely was there any agitation.

Two chambers were used, both of the design shown diagrammatically in Fig. 1. The outer cover was made of sheet iron in one (volume, 34 l.), copper in the other (volume, 22 l.). Wet- and dry-bulb thermometers and an air stirrer were mounted in the top of the cover, which could be lowered over the housing for the pig, making a gas-tight liquid-paraffin seal in a base tray. The pig rested on 16-gauge galvanized iron wire in the form of a $\frac{3}{8}$ in. (10 mm) mesh, with a similar mesh above the animal. On this second mesh, and immediately below the air stirrer, was a polythene tray containing soda asbestos (British Drug Houses 3–6 mesh), which was used to absorb carbon dioxide and water. The carbon dioxide concentration in the chamber was nearly always below 0·1 % and always below 0·2 %; the relative humidity was held below 50 % and in any one experiment showed only small variations during any period over which oxygen consumption was being measured. As the pig used oxygen, and carbon dioxide was absorbed, so oxygen entered the chamber from a balanced spirometer; oxygen consumption could thus be measured directly as millilitres of dry gas at s.t.p. Corrections based on the volume of the chamber were made for any differences in temperature, humidity and pressure between readings.

One chamber was mounted in a cupboard in which the temperature could be held at any desired level between room temperature and 38° C, and another in a large refrigerator which was used to produce an environmental temperature in the region of 4° C. A series of observations on one pig was usually begun at 30° C, after which the temperature was changed upwards or downwards and oxygen consumption determined at different temperatures, returning to 30° C for a final determination. Measurement of oxygen consumption at any one temperature was always preceded by an equilibration period, usually 20–30 min; this was important if misleading readings were to be avoided. The equilibrium period during which readings were taken for any given temperature was never shorter than 30 min.

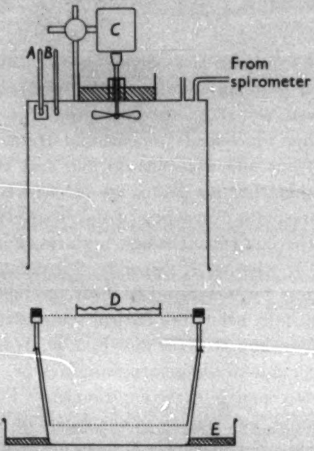

Fig. 1. Diagram of apparatus used for measurement of oxygen consumption. When in use the bottom rim of the cover rests in the oil seal in the base tray. Cover diameter 35 cm. *A*, wet-bulb thermometer; *B*, dry-bulb thermometer; *C*, stirrer motor, with oil seal and fan; *D*, soda asbestos; *E*, base tray with oil seal.

At the end of the experiment the rectal temperature and body weight were again recorded. In some cases the carbon dioxide produced by the pig was measured. To do this the soda asbestos used as absorbent was weighed before the experiment, then afterwards washed quantitatively into water, dissolved, and made to a known volume. Soda asbestos goes into solution leaving a small amount of brown insoluble residue. The carbon dioxide content of the solution was then estimated on the manometric van Slyke apparatus, and after deduction of the corresponding initial carbon dioxide content of the soda asbestos before the experiment, a figure was obtained for carbon dioxide production during the experiment.

The air-stirring fan was inside the chamber but the motor driving it was placed outside, so that it would not contribute heat to the chamber. The drive from motor to fan passed through a liquid-paraffin seal in the lid of the cover. To test the efficacy of this seal and of the seal made by the cover in the base tray, air containing 1·60% carbon dioxide was introduced into the chamber; under operating conditions, but without carbon dioxide absorber or animal, there was no detectable change in this concentration over a 1 hr period, using the Haldane apparatus for the gas analysis. It was, therefore, considered that the seals were adequate to prevent loss of carbon dioxide, since the concentration never rose above 0·2% in any of the experimental periods. The concentrations of oxygen to which pigs were exposed for these experiments lay between 18 and 22%.

RESULTS

Oxygen consumption

Oxygen consumption was measured on eighteen animals over the approximate temperature range 20–38° C and on eighteen animals at two temperatures in the region of 23 and 4° C.

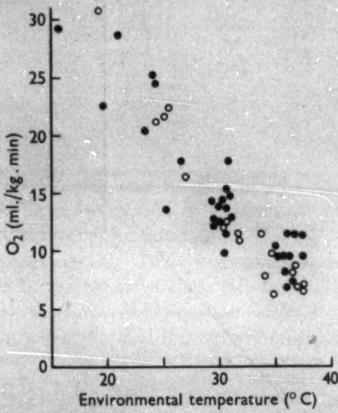

Fig. 2. Relation between environmental temperature and oxygen consumption for 8 Large White pigs 10–18 hr of age. ○, not suckled; ●, suckled.

Environment of 20–38° C. Figure 2 shows the relation between rate of oxygen consumption per unit body weight and environmental temperature over the range 20–38° C for Large White pigs aged less than 1 day at the time of experiment. Figure 3 gives corresponding results for pigs over 1 day but less than 1 week in age. It is apparent that there is a marked rise in oxygen consumption in response to fall in temperature in both age groups. In Fig. 4 these two sets of results are plotted together, and mean values calculated for a temperature of 30° C are inserted for each group with the corresponding linear regression lines. With the units 'ml. oxygen/kg. min' and ° C, the average regression of oxygen consumption on temperature is $-1{\cdot}08$ for the pigs less than 1 day old, and $-1{\cdot}02$ for pigs 1–6 days old. There is no significant difference between these regressions, which have as mean value $-1{\cdot}05$; the mean correlation coefficient is $-0{\cdot}93$.

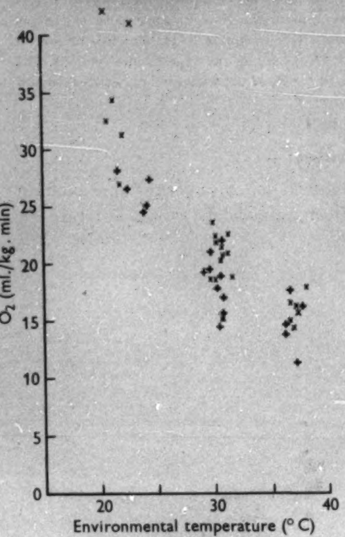

Fig. 3

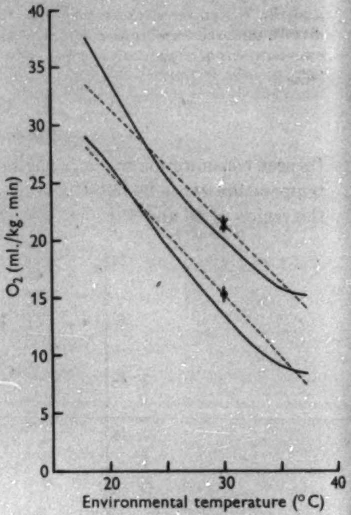

Fig. 4

Fig. 3. Relation between environmental temperature and oxygen consumption for 10 Large White pigs 1–6 days old. ×, 1–2½ days old; +, 3–6 days old.

Fig. 4. Relation between environmental temperature and oxygen consumption for Large White pigs in the first week of life. The lower curve refers to pigs 10–18 hr old, the upper curve to pigs 1–6 days old. ●, calculated mean oxygen consumption at 30° C for pigs aged 10–18 hr; ×, corresponding mean for pigs aged 1–6 days. Standard errors are shown as ordinates for each mean. Interrupted lines = regression lines; continuous lines = average curves.

However, there is a highly significant difference ($P < 0{\cdot}001$) between the means for the two age groups. At 30° C, these means are 15·22 and 21·33 ml. oxygen/kg. min for the younger (< 1 day) and older (1–6 days) groups respectively, each with a standard error of 0·67. Division of the older pigs into those aged 1–2½ and those aged 3–6 days shows that there is no significant difference between these two older groups. The change in rate of the pig's oxygen consumption, therefore, takes place between the first and second days of life. Three of the animals under 1 day old had not been previously suckled by the sow when they came to experiment. There was no evidence that they responded differently from pigs which had been suckled.

To test the adequacy of a 30 min period in representing the oxygen consumption of a pig at a given environmental temperature, estimates were made of oxygen consumption at 30° C at the beginning and end of experiments lasting between 3 and 4 hr. For pigs under 1 day of age the average rate of decline of oxygen consumption (6 experiments) was 0·40 ml./kg. min per hour, and for pigs 1–7 days of age the average rate of decline (6 experiments) was 0·07 ml./kg. min per hour. With mean initial levels of consumption of 15·22 and 21·33 ml./kg. min respectively, these rates of decline represent falls of 2·7% per hour and 0·3% per hour for the younger and older age groups. Similar rates of fall have been found in experiments at an environmental temperature of 20° C up to 9 hr in duration.

TABLE 1. Oxygen consumptions of new-born Large White pigs at two temperatures near 23 and 4° C. Both temperature and consumption are given as means with standard errors (6 animals in each age group)

Age (days)	Environmental temperature (° C)	Oxygen consumption (ml. dry gas s.t.p./kg. min)
Less than 1	22·8±0·4	21·6±1·1
	3·1±0·1	34·5±1·1
	22·2±0·1	24·9±1·0
2–4	23·3±0·9	25·7±0·9
	3·8±0·9	38·8±0·9
	23·0±0·2	26·0±0·6
5–6	24·1±0·6	22·7±0·6
	5·0±0·5	35·9±1·2
	23·6±0·5	22·9±1·0

Environments of 23 *and* 4° C. Table 1 summarizes oxygen consumption measurements made in the region of these two temperatures. For all three age groups there is a marked and highly significant ($P < 0{\cdot}001$) increase in oxygen consumption when the environmental temperature is reduced from near 23 to about 4° C, and consumption falls when the pigs are returned to the higher temperature. This result has been reported previously (Mount, 1958*c*). The rate of rise of oxygen-consumption-rate with fall in temperature is, however, less in these pigs than it is in the 20–38° C group. The mean rate of rise per 1° C fall in temperature between 23 and 4° C is 0·67 ml./kg. min.

The increase in oxygen consumption between under 1 day and 2–4 days (Table 1) is not as great with this group as it is in those used in environments of 20–38° C. This is because the 23 and 4° C group under 1 day old included four pigs over 20 hr old and two of 2 hr old. The ages of pigs in the 20–38° C group under 1 day old were more uniformly spread, and had an average age which was lower than that of the 23 and 4° C group.

Respiratory quotient and heat production

The mean respiratory quotient calculated from ten experiments, each of 5–9 hr duration, was 0·84 (S.E. ± 0·01). The calorie equivalent of oxygen at this R.Q. is 4·824 kcal/l. (Weir, 1949). On this basis the pig during the first day of life is producing, at an environmental temperature of 30° C, 106 kcal/kg.day (≡ 15·22 ml. oxygen/kg.min.) and 148 kcal/kg.day (≡ 21·33 ml. oxygen/kg. min) during the remainder of the first week. These values are in accordance with extrapolation from several sources quoted by Brody (1945), showing the relation between heat production and body weight for pigs 6 weeks of age and older.

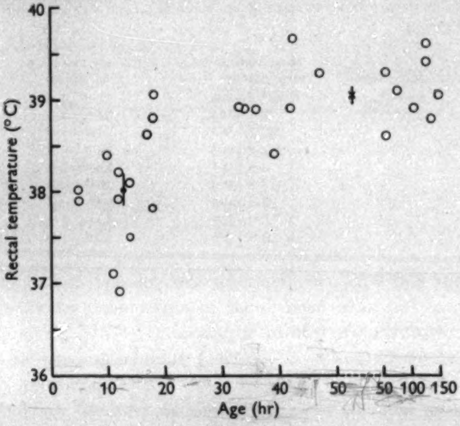

Fig. 5. Relation between age and rectal temperature for new-born Large White pigs. ●, mean value for first day of life; ×, mean value for pigs 1–6 days old; standard errors shown as ordinates for both means. Each point refers to one pig. Note condensed scale 50–150 hr.

Rectal temperature

Environment of 20–38° C. The relation between age and rectal temperature at the beginning of the experiment is shown for this group of pigs in Fig. 5. From a mean value of 38·0° C (S.E. ± 0·2) during the first day of life the rectal temperature rises to 39·0 °C (S.E. ± 0·1) for the remainder of the first week; the difference between these two values is significant ($P < 0·001$). Thus the change in rectal temperature proceeds coincidentally with the change in oxygen consumption.

Over this range of environmental temperature the rectal temperature rose during the course of an experiment at an average rate of 0·004° C/hr for pigs under 1 day of age, and 0·03° C/hr for pigs between 1 day and 1 week of age (9 pigs in each group, experimental duration from 3 to 9 hr). The difference between initial and final rectal temperatures was therefore small.

Environment of 23 *and* 4° C. Table 2 gives initial rectal temperatures and changes in rectal temperature with environment in pigs under 1 day and between 1 day and 1 week of age. The mean initial rectal temperature is higher than the 38·0° C value quoted previously as the mean value for pigs under 1 day old, owing to the different age composition of the group, as noted above. Exposure of these pigs to an environmental temperature of 4° C for 45 min produced falls in rectal temperature ranging from 0·7 to 2·7° C, with a mean fall of 1·7° C. In the older pigs the change ranged from a fall of 1·6 to a gain of 0·3° C, with a mean fall of 0·3° C.

TABLE 2. Changes in rectal temperature from the initial value when Large White pigs are exposed to environmental temperatures near 23 and 4° C. Temperatures are given as means with standard errors (6 animals in each age group)

Age (days)	Initial rectal temperature (° C)	Changes (° C) in rectal temperature during 45 min periods at mean temperatures of		
		23° C	4° C	23° C
Less than 1	38·6 ± 0·1	−0·4 ± 0·1	−1·7 ± 0·3	0·9 ± 0·2
2–4	39·1 ± 0·2	−0·3 ± 0·02	−0·6 ± 0·2	0·2 ± 0·2
5–6	39·5 ± 0·3	−0·4 ± 0·1	0 ± 0·1	−0·2 ± 0·1

Comparison of breeds

No significant differences were found between Large White and Landrace pigs in these experiments in respect of oxygen consumption and rectal temperature in relation to environment.

DISCUSSION

Under the conditions of these experiments, the new-born pig in the first week of life shows a vigorous metabolic response to cooling of the environment within the range 4–38° C. The measurement of the animal's oxygen consumption as the criterion of this response has shown that for the environmental temperature range from 20 to 38° C a fall of 1° C brings about a mean increase in oxygen consumption of 1·05 ml./kg. min and for the range from 4 to 23° C the corresponding increase is 0·67 ml./kg. min.

Increased metabolism on lowering of environmental temperature has been observed from 1 hr of age onwards, and it has taken place without exception in all the pigs used in this work. The initial conclusion is, therefore, that the new-born pig shows the metabolic response to external cooling characteristic of the adult homoeotherm. In this respect it resembles the human infant (Day, 1943); another resemblance lies in the lack of peripheral insulation in the form of fat or an effective hair covering.

The level of oxygen consumption at any given temperature, however, increases with age during the first 48 hr of life; this is shown in Fig. 4. The mean increment in oxygen consumption is 6·6 ml./kg. min, leaving the regression of oxygen consumption on environmental temperature unchanged. This rise occurs simultaneously with a rise in rectal temperature of 1° C, from 38 to 39° C (Fig. 5). Roy, Huffman & Reineke (1957) have measured metabolic rate in the calf in the new-born period, and find that heat production increases after birth to a maximum between the second and fourth days of life, and then falls rapidly until the eighth day, after which it falls more slowly. They made their observations in environmental temperatures ranging from 7 to 18° C and recorded a maximum heat production rate of 83 kcal/m^2. hr; this may be compared with the values given in Fig. 6 for other species. Body temperature reached its highest value also on the second day of life. In their work on the human infant, Cross, Tizard & Trythall (1957) found that oxygen consumption rose significantly during the first 3 days of life.

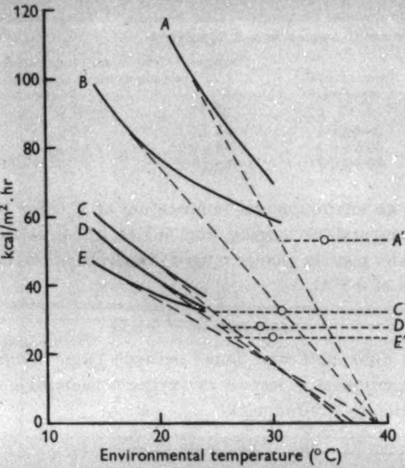

Fig. 6. Relation between heat production per unit of body surface and environmental temperature for the new-born pig (*AA'*); with added figures from results of Cairnie & Pullar (1957) on 4 kg pigs (*B*) and Herrington (1940) on mice (*CC'*), rats (*DD'*) and guinea-pigs (*EE'*). The average heat production rates are indicated by continuous lines; interrupted lines are drawn at a tangent to these to intersect the temperature axis at the corresponding rectal temperature. The horizontal interrupted lines indicate minimum metabolism observed; the open circles indicate the estimated critical temperatures.

The rise in body temperature found in these new-born pigs in the first 48 hr of life is in accordance with the findings of Newland, McMillen & Reineke

(1952) on the rectal temperature of the pig in the first few days after birth; after an initial drop during the 20 min after birth, they found that the animal's temperature rose to its final level in 2–10 days, both the extent of the initial fall and the rate of the subsequent rise being dependent on the environment. In the present work it was found that if at any time during the first few days of life the pig should be exposed to an environmental temperature of 4° C, the rectal temperature falls in spite of a marked increase in heat production with shivering; by the end of the first week, however, this fall no longer takes place when the animal is put under comparable conditions (see Table 2; cf. Holub, Forman & Ježková, 1957). This development of stability of deep body temperature with age has also been reported by McIntyre & Ederstrom (1958) for the young dog; they found in this animal a lag in the development of heat conservation as compared with heat production, so that as a result of a lowered environmental temperature the rectal temperature fell in spite of increased heat production.

If heat loss from the new-born pig were to occur in accordance with Newton's law of cooling, and without change in peripheral insulation, it would be expected that with a rise in deep body temperature the increase in oxygen consumption, as a direct measure of the rate of heat production, would be a constant proportionate increase over the whole range of environmental temperature. This, however, is approximately true only over the lower part of the temperature scale from 30° C downwards. Above 30° C the rise in oxygen consumption with rectal temperature is greater than would be expected from the law of cooling, suggesting a decreased thermal insulation. A postural change may contribute to this; in the cold the pig is in a crouching position, whilst in warm conditions it lies stretched out, with consequent increased effective surface area from which loss of heat can occur.

These oxygen consumption values may be expressed as heat production per unit surface area, using the formula quoted by Brody (1945) for surface area of the young pig (surface area in $m^2 = 0{\cdot}097 \times (\text{weight in kg})^{0{\cdot}633}$). Calculated in this way the surface area of a pig 4 days old, weighing 1·5 kg, is 0·125 m^2 and at an environmental temperature of 30° C the heat production rate is 74·1 kcal/m^2.hr. For each 1° C fall in temperature over the range 20–38° C the mean increment in heat production is 3·55 kcal/m^2.hr. This is approximately double the increments estimated from figures obtained by Herrington (1940) from fasting mature rats, mice and guinea-pigs over an environmental temperature range from 10 to 35° C; below the zone of thermal neutrality for each species his results show that, for each 1° C fall in temperature, heat production increased by 1·8, 1·9 and 1·1 kcal/m^2.hr for rats, mice and guinea-pigs respectively. Herrington's experiments were carried out on animals after a fasting period; to use fasting new-born pigs would not give stable or readily repeatable conditions on account of the animal's tendency to become hypoglycaemic and comatose and finally to die when deprived of food for relatively short periods (Morrill, 1952; Goodwin, 1957). On this account standard conditions for the pig were sought instead by withholding food during the course of the experiment.

Cairnie & Pullar (1957) made observations on pigs weighing from 4 to 12 kg in a gradient layer calorimeter; their pigs differed from those used in the present work in being heavier and older, in being fed *ad libitum* during the period of measurement, and in being acclimatized to the temperature at which the measurement was made. From their results on the 4 kg pigs, that is those most nearly comparable, over the environmental temperature range 15–30° C, it may be calculated that metabolism rose by 2·4 kcal/m^2.hr for every 1° C fall in temperature.

These figures are expressed graphically in Fig. 6, and in addition the minimum values observed for the new-born pig and Herrington's rats, mice and guinea-pigs are shown by horizontal interrupted lines. The minimum value of heat production per unit body surface in the new-born pig is approximately twice that in Herrington's animals. Also in Fig. 6 average curves for heat production against environmental temperature are shown as continuous lines, and the averaging is carried only up to a point 5° C below the critical temperature in each case. The results may then be compared on the basis put forward by Scholander, Hock, Walters & Irving (1950), and discussed by Burton & Edholm (1955) and by Hart (1957), which postulates that straight-line extrapolation from values below the critical temperature cuts the environmental temperature axis at the deep body temperature. This would be expected in the ideal case where the deep body temperature is maintained constant below the critical environmental temperature by an elevated heat production, in accordance with Newton's law of cooling, without further increase in insulation, which is already maximal when the decreasing temperature has reached the critical temperature. Such extrapolations are drawn in Fig. 6 and appear as tangents to the heat production–temperature curves for the different groups of animals; it is obvious that over this temperature range the hypothesis applies only approximately. The higher minimum level of metabolism, when taken with rectal temperature, does, however, indicate the relatively smaller degree of insulation per unit of surface area in the new-born pig as compared with the adult rat, mouse and guinea-pig, and the greater increase in metabolism with fall of temperature which occurs when body temperature remains constant.

This comparison is made between the pig at an average time of 3 hr after food, and Herrington's rats, mice and guinea-pigs at average times of 21, 15 and 27 hr after food respectively. The effect of a longer period of fasting on the pig is to reduce heat production and body temperature, but the values of Fig. 2 on unsuckled pigs suggest that a fasting period up to approximately 12 hr makes little difference. The comparison made here between the new-born pig and fasting rats, mice and guinea-pigs is valid, however, in respect of comparing thermal insulations if deep body temperatures are considered at the same time.

The most convenient way of doing this is to calculate conductance values using Herrington's (1940) equations, which express essentially the heat loss per unit area divided by rectal–surface, surface–air and rectal–air temperature differences. In units comparable with his, and at an environmental temperature of 26·7° C which he used for the calculation, the 4-day-old 1·5 kg pig produces 2062 kcal/m^2. 24 hr, has a rectal temperature of 39·0° C, and a mean skin temperature of 36·9° C. This skin temperature is derived from measurements on six pigs with thermocouples at five sites on the body surface simultaneously over an environmental temperature range of 20–38° C; the mean of the individual differences from individual rectal temperatures is then subtracted from 39·0° C to give the value 36·9° C. The three conductances calculated from these data are shown in Table 3, together with Herrington's values for rats, mice and guinea-pigs; evaporative loss is assumed as 15% of total heat loss. It is apparent that the rectal–surface conductance of the new-born pig is approximately 8 times that of the smaller furred mammals, and the other conductances are also higher. That this is a demonstration of the insulative property of fur is borne out by Herrington's (1940) estimation of the true skin temperature of the guinea-pig as 35·2° C, as distinct from the temperature at the outside of the fur, which he found to be 31·1° C (at an environmental temperature of 26·7° C). The guinea-pig deprived of fur would thus have conductances approaching those of the new-born pig.

TABLE 3. Calculated thermal conductances for the new-born pig compared with Herrington's (1940) figures for rat, mouse and guinea-pig

	Conductance, kcal/m^2. hr per ° C		
	Rectal–surface	Surface–air	Rectal–air
New-born pig	984	172	146
Mature rat	123	151	68
Mature mouse	162	162	81
Mature guinea-pig	91	142	55

Correlated with these high conductances, the critical temperature for the new-born pig appears to lie in the region of 34–35° C, that for the other animals being approximately 30° C. Thus when environmental temperature falls heat production rises in the pig significantly earlier and at a greater rate than it does in the other species. The general conclusion is that in the new-born pig the rectal temperature is maintained by a variation of heat production dependent on the environmental temperature with very little apparent contribution from either magnitude or control of thermal insulation.

SUMMARY

1. The mean oxygen consumption of pigs in the first day of life, at an environmental temperature of 30° C, was 15·22 ml./kg.min.

2. This value rose to 21·33 ml./kg.min during the first 48 hr, and this change was accompanied by a rise in rectal temperature from 38·0 to 39·0° C.

3. Oxygen consumption increased by an average of 1·05 ml./kg.min for each 1° C fall in environmental temperature over the range 20–38° C, and by 0·67 ml./kg.min over the range 4–23° C.

4. There was an apparent critical temperature at 34–35° C; the calculated thermal conductance was correspondingly high.

5. It is concluded that the new-born pig responds to cooling of the environment by a marked increase in heat production, which maintains rectal temperature with little contribution from thermal insulation.

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PROPERTIES OF SPINAL INTERNEURONES

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Several studies of internuncial neurones (interneurones) in the spinal cord have been made. Lloyd (1942) recorded discharge in such cells related to long spinal reflex activity, and Renshaw (1946) studied the activity of interneurones activated by antidromic impulse conduction in motoneurones. After the introduction of the technique of intracellular recording by micropipettes, Woodbury & Patton (1952), Eccles, Fatt & Koketsu (1954), Frank & Fuortes (1955), and Suda, Koizumi & Brooks (1958) have made observations on interneurones as well as other elements within the cord. The most systematic studies of unitary responses of interneurones have been those of Frank & Fuortes (1956*a*), Kolmodin (1957) and Haapanen, Kolmodin & Skoglund (1958).

From the above studies certain general features of interneurones have been characterized. Among these are the frequent occurrence of repetitive discharge to single afferent volleys, the presence in many units of spontaneous or background discharge and the frequent occurrence of convergence from multiple afferent sources on individual interneurones. Frank & Fuortes (1956*a*) emphasized the considerable variation in behaviour among different interneurones and the fact that information about them is limited, although they were able to establish certain general features of their response patterns. Both Frank & Fuortes (1956*a*) and Haapanen *et al.* (1958) described certain characteristic features of membrane and action potentials of interneurones recorded by intracellular micropipettes. Both groups described responses to nerve volleys and to natural stimulation. Kolmodin's study (1957) was concerned principally with the responses of interneurones to stretch-evoked afferent discharge from muscle. Eccles *et al.* (1954) investigated principally the activity of interneurones activated by collaterals of motoneurones.

The present study is concerned with further characterization of the properties and functional organization of interneurones in the lumbosacral cord of the cat. This paper is devoted principally to the general properties of such cells; subsequent papers deal with response patterns to afferent input from various sources and the responses to natural stimuli.

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METHODS

Adult spinal cats were used, acutely decapitated under preliminary ether anaesthesia (as described by Hunt & Paintal, 1958). After lumbosacral laminectomy and limb dissection, exposed tissues were covered by pools of paraffin oil equilibrated with O_2 95% + CO_2 5%. Body temperature was usually maintained between 36 and 38° C. For intracellular recording glass micropipettes filled with 3 M-KCl were used. Relatively high-resistance electrodes were found necessary to minimize damage to interneurones. Satisfactory electrodes usually had resistances in excess of 30 MΩ. These were connected by a cathode-follower probe, using an electrometer tube (Raytheon 5886) leading to a modified transistor d.c. amplifier of the type described by MacNichol & Bickart (1958).

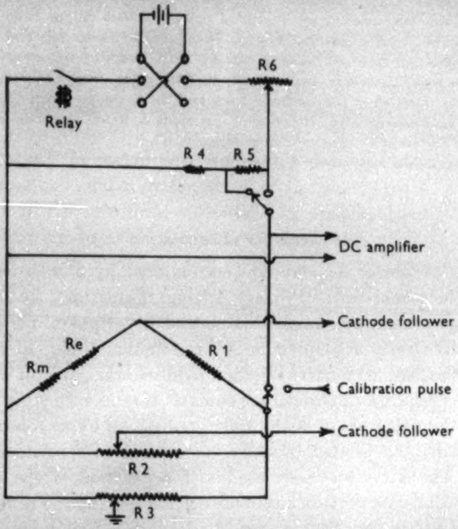

Fig. 1. Schematic view of recording and stimulating circuit. R1 = 500 MΩ, R2 = 10 kΩ, R3 = 10 kΩ, R4 = 100 Ω, R5 = 1000 Ω, R6 = 10 kΩ, Re = electrode resistance, Rm = membrane resistance. Relay is Clare mercury wetted type.

In many experiments utilizing intracellular recording, the same electrode was used for passing current through the cell and for recording. For this purpose a modification of Araki & Otani's (1955) bridge circuit was used. The final version of the circuit used is shown in the schematic diagram of Fig. 1. This system permitted measurement of electrode resistance, an approximation of the effective recording time constant and correction thereof by capacitative feed back, and the passage of current through the cell. The time constant, when corrected, was less than 50 μsec.

Electrode resistance was estimated from the magnitude of the recorded signal when a known calibrating pulse was introduced. The effective recording time constant was corrected by increasing capacitative feed back until the recorded response to a rectangular calibrating pulse approximated the original wave form. Estimation of current passed through the micro-electrode was made by measurement of the voltage applied across the bridge through the known (500 MΩ) grid leak resistance and estimated electrode resistance.

Spinal interneurones appear to be much more easily damaged by movement relative to the micro-electrode tip than do motoneurones. Hence prevention of movement is a prime problem. Muscle contraction was avoided by administration of tubocurarine or occasionally of Flaxedil (gallamine triethiodide; May and Baker). Spinal cord fixation was achieved by paired pins in the ilia, a dorsal spinous process clamp just above the laminectomy site, and by two pairs of rigid steel angulated members grasping the vertebral bodies near the recording site. The array of fixing devices was attached to a heavy aluminium base which also held the micromanipulator. Respiratory movements were minimized by using artificial ventilation by a Palmer Ideal pump at rapid rate (44 min) and low tidal volume (30–40 ml.). In most experiments bilateral pneumothorax was also employed.

Recording sites were known only approximately from the point of entry of the micro-electrode into the cord, the direction of the electrode track and the depth to which the electrode was inserted by the micromanipulator. No attempt was made to locate electrode tracks by histological methods. The majority of units were in segments L7–S1.

RESULTS

Criteria for identification

Several types of neural elements are encountered by a micro-electrode inserted into the spinal cord. Primary afferent fibres may be identified by single impulses evoked by stimuli to a particular peripheral nerve, when the latency can be clearly accounted for by conduction time. Identification is aided, when the peripheral nerve is intact, by discharge patterns characteristic of peripheral receptors. Motoneurone somata were identified on the basis of inflexion on the rising phase of the spike, post-spike hyperpolarization, synaptic potentials, and limited capacity to respond to repetitive antidromic stimulation. The latter was associated with separation of the impulse into two distinct components (Brock, Coombs & Eccles, 1953; Frank & Fuortes, 1955). Motor axons were distinguished by impulses of singular configuration evoked by antidromic stimulation, a capacity to respond to such stimulation at high frequencies, lack of prominent post-spike hyperpolarization, and synaptic potentials of much smaller amplitude than in motoneurone somata.

Most of the remaining units were classed as interneurones. In general, the latter could be easily distinguished from the other types of neurones mentioned by their repetitive response to volleys in more than one peripheral nerve (convergence) and their lack of a directly conducted response to antidromic stimulation of ventral root fibres. Our criteria for identification were generally similar to those used by Frank & Fuortes (1955). An attempt was also made to distinguish between recording sites in interneurone somata and the axons (see below, p. 349).

When glass micropipettes were used, elements were encountered which showed steady membrane potentials but no response to nerve volleys. Frank & Fuortes (1955) reported similar findings. In some cases direct stimulation via the same micropipette as that used for recording evoked an impulse. In others direct stimulation failed to produce a spike. The latter may have been non-neural elements. Non-responsive elements of both types were classed as unidentified.

Recording from interneurones

Recording from interneurones has been more difficult than from motoneurones. Many units were impaled only transiently as a micropipette was advanced into the cord, the abrupt appearance of a resting membrane potential being followed by a series of injury discharges and rapid decline of the membrane potential. Relatively few units could be held for long periods with stable membrane potentials. With KCl-filled micropipettes variations in potential were seen during advance along a track, some of which were abrupt and clearly associated with entry into a cell. However, slower variations, usually 10–20 mV in magnitude, were often observed without evidence of cell penetration but related to movement of the micropipette. Such potential changes probably result from alterations in junctional potential and were associated with measurable changes in electrode resistance (cf. del Castillo & Katz, 1955; Frank & Fuortes, 1955). Potentials occurring with apparent cell impalement varied widely in magnitude from about 20 mV to nearly 100 mV but were usually in the range of 40–60 mV. In view of the difficulties in impaling interneurones without obvious damage and the variations in potential due to the micropipettes themselves, no reliable estimate can be given as to the normal level of membrane potential in interneurones (cf. Frank & Fuortes, 1955; Haapanen *et al.* 1958).

Spike potentials recorded with KCl-filled micropipettes showed considerable variation. On approaching a cell spike potentials could often be recorded in the absence of a recorded membrane potential. These were usually diphasic in positive–negative sequence (cf. Haapanen *et al.* 1958), although at times impulses of simple monophasic positive configuration were seen. After impalement spikes appeared as positive deflexions. In most units spike amplitude approached or exceeded the size of the recorded resting membrane potential. However, certain interneurones showed spike potentials far smaller than the resting membrane potential, often with a relatively slow falling phase of approximately exponential form. In such cases the membrane in the region of the micro-electrode, because of injury, apparently does not undergo the active regenerative changes associated with the impulse, but the spike is recorded electrotonically at some distance.

An attempt has been made to distinguish between recording sites in interneurone somata and axons. Recordings in which synaptic potentials a few millivolts or larger were observed were considered to be from interneurone somata. In contrast, recordings which displayed very small synaptic potentials or no detectable synaptic potentials were considered to be from interneurone axons (Fig. 2). With the high-resistance micro-electrodes used in the present study, primary afferent fibres, whose conduction velocities indicated diameters measurably less than 10 μ, have been impaled. Therefore it is thought that there is no *a priori* reason why interneurone axons should not have been impaled with the same sized electrodes. Haapanen *et al.* (1958) suggested that they were unable to impale primary afferent fibres smaller than 10 μ in diameter and hence they doubted whether interneurone axons

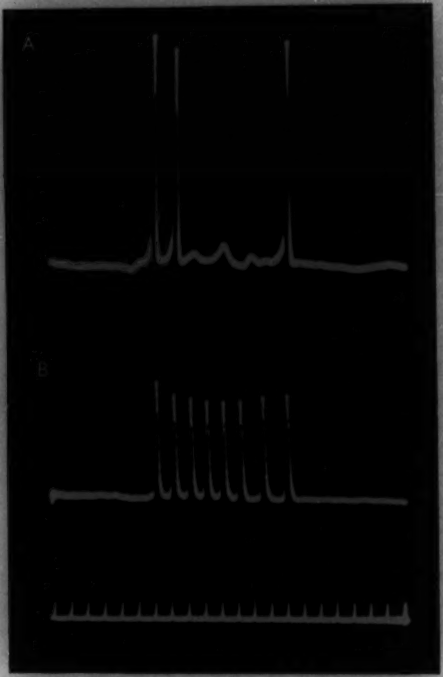

Fig. 2. A, recording from site considered to be interneurone soma; response to volley in plantar nerve. Note synaptic potentials; spike potential 77 mV. B, recording from site presumed to be interneurone axon; response to deep peroneal nerve volley; spike potential 56 mV. Note lack of detectable synaptic potentials. Time marker, 1 msec.

could be impaled. However, their evidence in this regard was not critical. Unlike motoneurone somata, interneurone somata show no conspicuous 'negative after-potential' and no prolonged after-hyperpolarization. However, a brief post-spike 'positivity' was frequently seen, similar to that observed in squid axon (Hodgkin & Katz, 1949), which may be a consequence of an increased potassium conductance following the spike in cells with a resting potential less than the potassium equilibrium potential. When repetitive response was initiated in interneurone somata the brief post-spike positivity that was frequently observed was followed by a slow depolarization leading to the next impulse. Such a slow depolarization was also seen in interneurones discharging spontaneously. In contrast, spike potentials recorded from interneurone axons usually rose from the base line without detectable synaptic potential and returned to the previous potential level without post-spike positivity. When presumably being recorded from interneurone somata, spike potentials have rarely shown an inflexion in the rising phase even in the case of high-frequency discharge and only on one occasion was it possible to separate the spike into two components by repetitive stimulation, as can be done in motoneurones (Brock *et al.* 1953; Frank & Fuortes, 1955). In some units, particularly when deterioration was evident, small spike potentials were seen, sometimes of two or three discrete amplitudes. In some cases spikes of two distinct sizes could be separately evoked by stimulation of two distinct peripheral sources and when coincident the spike amplitudes summed. In such cases it is presumed that the small spikes are recorded electrotonically from a distance, perhaps from different parts of the cell.

TABLE 1. Spike duration of spinal neurones, directly stimulated (msec)

	Interneurone		Motoneurone		
	Soma	Axon	Soma	Axon	Primary afferent fibre
	0·65	0·65	1·11	0·52	0·37
	0·38	0·30	0·88	0·47	0·45
	0·38	0·50	0·99	0·44	0·37
	0·67	0·50	1·06	0·48	0·39
	1·00	0·36	0·72	0·69	0·50
	0·39	0·41	0·98	0·65	0·42
	0·48	0·72	1·20	0·61	0·47
	0·39	0·35	—	—	—
	0·28	0·34	—	—	—
Mean	0·51	0·46	0·99	0·55	0·42

Absolute values of spike potential amplitude cannot be given with assurance, in view of the uncertainty of resting membrane potential values. In some units overshoot during the spike was observed, in others not. Frank & Fuortes (1955) and Haapanen *et al.* (1958) reported similar findings. The latter authors noted overshoot in 10% of the interneurones examined. Table 1 presents representative findings on spike duration in interneurone somata and axons and in motoneurone somata and axons. No clear difference has been seen between the duration of spikes from recording sites considered to be in somata and those considered to be in interneurone axons. On the other hand, the spike duration of the interneurone soma appears to be appreciably shorter than that of the motoneurone soma. Our results differ from those of Frank & Fuortes (1955), who considered the spike duration of impulses from interneurone somata to be longer (*ca.* 1 msec) than those from interneurone axons (0·3 msec).

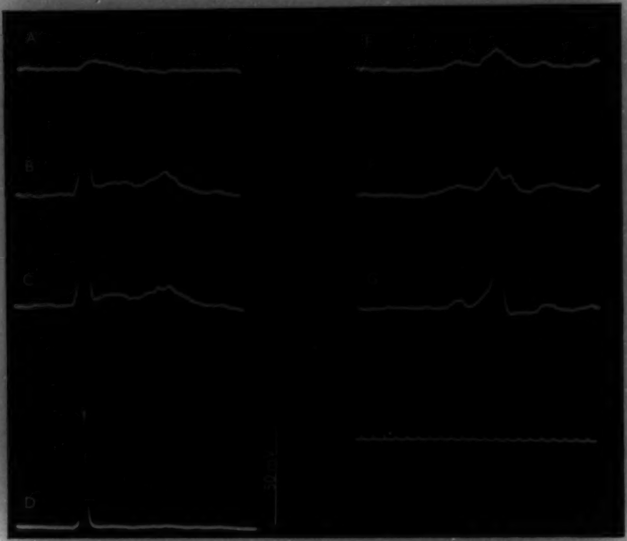

Fig. 3. Synaptic potentials of interneurone soma. A–D are responses to deep peroneal volleys; stimulus strength increased from A to C; D same strength as in C. E–G are responses to sural volleys, stimulus strength increased from E to G. Amplification in A–C and E–G was 5 times that in D; stimuli at beginning of sweeps; time marker, 1 msec.

Synaptic potentials

Figure 3 illustrates some features of synaptic potentials recorded from an interneurone soma. In A–D the responses to volleys in the deep peroneal nerve are shown (A–C being at 5 times the amplification of D). Stimulus strength was successively increased from A to C. In A an excitatory synaptic potential is evoked which is subthreshold for initiation of an impulse; this has a fairly simple wave form and decays to half amplitude in about 2·4 msec.

However, the excitatory synaptic potential was usually more complex (as in E and F) and showed components which decayed more rapidly. Although in the latter records contamination by inhibitory actions cannot be excluded, it seems likely that the synaptic potentials shown result from prolonged synaptic action, probably due to summation of temporally dispersed synaptic events. In view of the effective time constant of the interneurone soma (see below), the rate of decay of the synaptic potential shown in A is much slower than would be expected from passive decay of membrane potential change to a brief synaptic action. It has not been possible to record excitatory synaptic potentials considered to result from brief synchronous presynaptic actions as can be done in motoneurones. Even with weak afferent volleys measurable excitatory synaptic potentials were obviously compounded of temporally dispersed events.

In Fig. 3B and C the excitatory synaptic potential evoked an impulse. This was followed by a maintained synaptic potential which failed to evoke another spike although the level of depolarization associated with the initiation of the first impulse was exceeded. A large and prolonged post-spike hyperpolarization that is so conspicuous a feature of motoneurone somata is obviously lacking in records from interneurone somata. Impulses initiated by direct stimulation were also not followed by prolonged hyperpolarization (see below, p. 361). The threshold for initiation of an impulse in the interneurone of Fig. 3 occurred when the cell was depolarized from 2·5 to 4 mV by the synaptic potential. The level of depolarization by the synaptic potential which was associated with impulse initiation ranged from 2·5 to 10·9 mV, the average value being close to that found in association with impulse initiation in motoneurone somata (Brock, Coombs & Eccles, 1952). In addition to the excitatory synaptic potentials already considered, synaptic potentials characterized by an increase in membrane potential and inhibition of interneurone responses were also observed, but these have not been systematically studied. Haapanen *et al.* (1958) have observed synaptic potentials in interneurones, both excitatory and inhibitory, and associated with depolarization and hyperpolarization respectively. They have studied in particular the synaptic potentials and firing levels in response to natural stimulation.

Responses to current pulses

Using the modified bridge circuit of Araki & Otani (1955) described in Methods, anodal or cathodal current pulses were passed through the same electrode as was used for recording. Measurements were made of the strength–latency relations by recording the latency to onset of spike potential and the current intensity. The time constant was derived from the following equation:

$$I_0/I = 1-e^{-t/\tau}$$

where I_0 is rheobasic current, I current, t latency, and τ the time constant

(Frank & Fuortes, 1956*b*). Figure 4 shows an example of recordings from an interneurone soma used for strength–latency measurements, and Fig. 5 a plot of the relation for this unit. Table 2 presents findings on the time constant of interneurones and other units encountered in the spinal cord. The time constant of interneurone axons was brief and comparable to that of primary afferent fibres and motor axons. The average time constant of interneurone

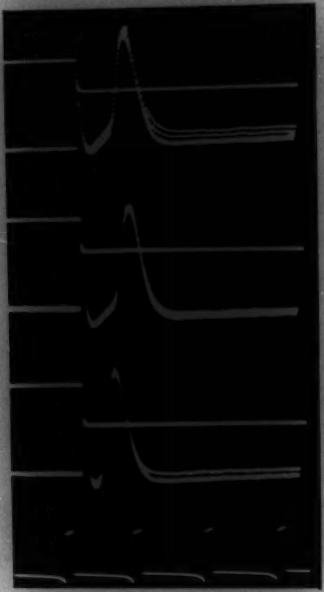

Fig. 4. Strength–latency measurements by direct stimulation of interneurone soma at three current strengths. Upper beam, current; lower beam, micro-electrode recording; time marker, 1 msec.

TABLE 2. Time constant (msec)

	Interneurone		Motoneurone		
	Soma	Axon	Soma	Axon	Primary afferent fibre
	0·80	0·14	0·66	0·28	0·25
	0·48	0·40	0·53	0·27	0·20
	0·69	0·19	0·74	0·22	0·13
	0·42	0·71	0·71	0·27	0·29
	0·27	0·29	0·59	0·42	0·24
	0·28	0·19	0·57	0·39	0·35
	0·26	0·20	1·48	0·42	0·34
	—	0·21	—	0·25	—
Mean	0·46	0·29	0·75	0·31	0·26

somata was longer than that of interneurone axons but shorter than that of motoneurone somata, but the data are too limited to have statistical significance. Since the only criterion for identification of recording site as interneurone soma was the presence of synaptic potentials of some magnitude, some of the recording sites classified as interneurone somata might have been from interneurone axons in close proximity to the cell body. For this reason, and because of the comparatively small number of observations, one cannot be certain that there is a significant difference between the time constant of an interneurone soma and that of a motoneurone soma. Average rheobasic current for interneurone somata was $2 \cdot 5 \times 10^{-9}$ A.

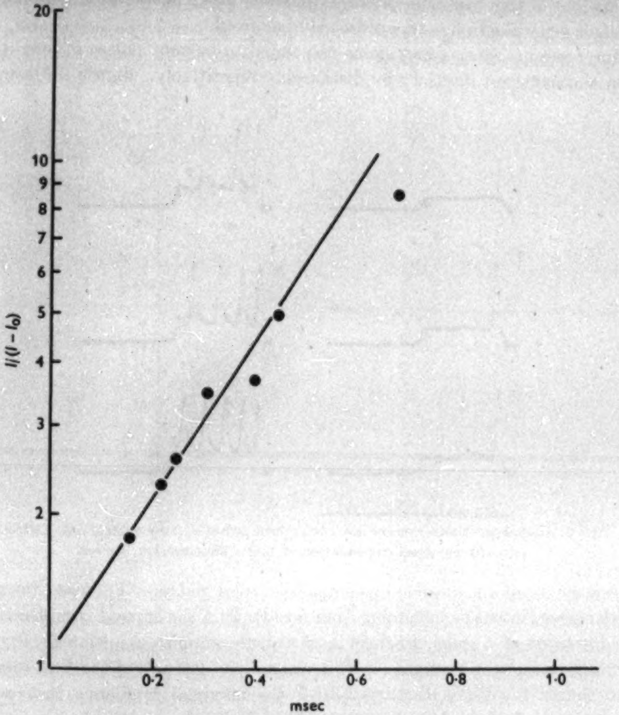

Fig. 5. Plot of relation between $I(I-I_0)$ and latency for unit of Fig. 4, where I = current, I_0 = rheobasic current. Semi-log. scale

The high resistance of electrodes needed to record from interneurones and the variation of electrode resistance with movement prevented measurement of the time constant in the direct manner employed by Araki & Otani (1955) for toad motoneurones, that is, by the time course of membrane potential change following the application or withdrawal of current. A further difficulty in the use of this technique was the presence of a considerable capacitative artifact when the bridge was resistively balanced.

Interneurone somata and axons responded to current pulses of long duration and sufficient intensity by discharging repetitively. Figure 6 illustrates the response of an interneurone soma to current pulses of 9·2 msec duration, with current intensity increasing from A to F. In A the current is subthreshold for initiation of a spike, whereas in B a single impulse is initiated. Further increase in current strength (C–F) decreased the latency of the first impulse and caused repetitive discharge. In F the maximal frequency reaches approximately 650/sec. It may be noted that the brief post-spike hyperpolarization, relative to the firing level, was followed by a slow depolarization leading to the next impulse (see especially D). The slope of the slow depolarization was the greater the shorter the impulse interval. The impulse frequency was highest between the first and second impulses and decreased slightly thereafter (F). Figure 7 shows the relationship between frequency of discharge of an interneurone soma during passage of an outward current pulse and the time after the first impulse. It may be noted that the impulse frequency decreases for successive impulse intervals. However, adaptation to prolonged current pulses was not great and maintained direct current was capable of

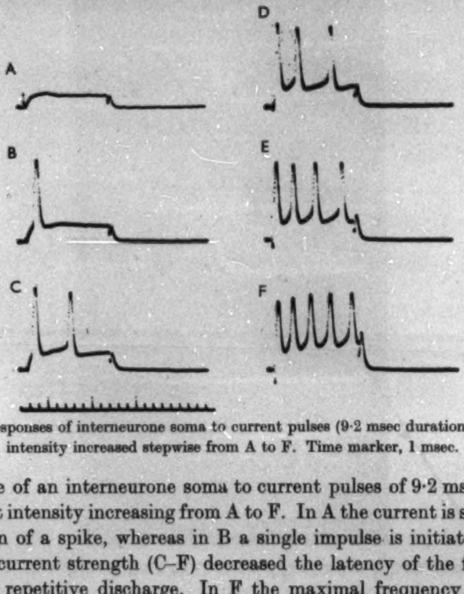

Fig. 6. Responses of interneurone soma to current pulses (9·2 msec duration). Current intensity increased stepwise from A to F. Time marker, 1 msec.

eliciting a continued high-frequency discharge for many minutes. Interneurone somata showed a greater tendency to repetitive discharge than did primary afferent fibres or motor axons. Motoneurone somata showed the least tendency to discharge repetitively, and even strong current pulses evoked only a low-frequency discharge. Figure 8 compares the repetitive discharges initiated in an interneurone soma and in a motor axon. In this plot the number of impulses initiated during a 14 msec current pulse is shown as a function of applied current. The slope of the relation is much greater for the interneurone than for the motor axon. In addition to the features described above, some interneurone somata showed oscillatory fluctuations in potential during passage of depolarizing current pulses, which were similar to those found in squid neurones by Hagiwara & Oomura (1958), but less marked.

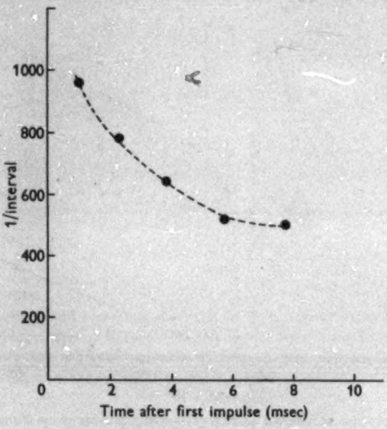

Fig. 7. Frequency of successive impulses in repetitive discharge set up by a prolonged constant current pulse.

Even strong current pulses, when made suitably brief, initiated only one impulse, which was not followed by detectable synaptic potential. Impulses were not initiated after the cessation of outward current pulses. On the other hand, on cessation of strong inward current pulses a single impulse was sometimes evoked, an effect analogous to anodal break excitation in axons. As noted above, impulses evoked by direct stimulation of interneurone somata were not followed by a conspicuous prolonged hyperpolarization.

Inward current pulses to interneurone somata caused interruption of spontaneous discharge and blocked initiation of reflexly evoked discharge, leaving only synaptic potentials evident. In recording sites considered to be from interneurone axons inward current of graded strength caused an increase in amplitude of spike potential and then, with further increase in strength, an abrupt decrease. In the latter case the hyperpolarization was apparently sufficient to block conduction of impulses into the region near the micro-electrode. Consequently only attenuated spikes were recorded by electrotonic spread from a distance.

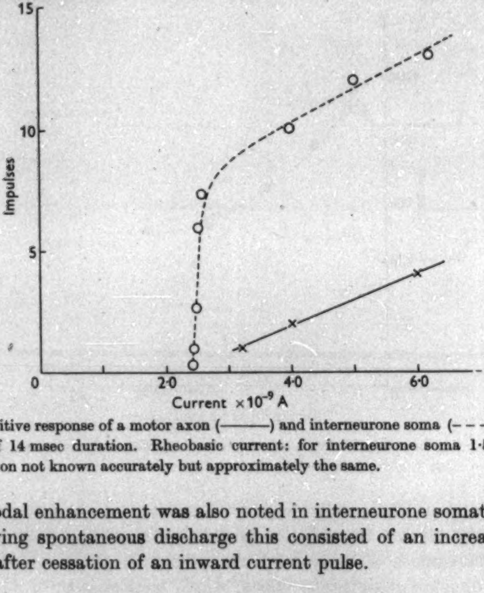

Fig. 8. Repetitive response of a motor axon (———) and interneurone soma (- - - -) to current pulses of 14 msec duration. Rheobasic current: for interneurone soma $1 \cdot 5 \times 10^{-9}$ A, for motor axon not known accurately but approximately the same.

Post-anodal enhancement was also noted in interneurone somata. In some units showing spontaneous discharge this consisted of an increased rate of discharge after cessation of an inward current pulse.

The mechanism of repetitive discharge

One of the striking features of the reflex response of spinal interneurones is the tendency for repetitive discharge to a single orthodromic volley. Of the various possible mechanisms for the production of repetitive discharge, two major types may be recognized: (1) self re-excitation of the cell by after-currents or by activity in reverberating circuits initiated by recurrent collaterals or axons of the same cell, and (2) prolonged synaptic excitation. Self re-excitation as the sole basis for repetitive discharge in interneurones may be ruled out by the fact that direct stimulation by a brief depolarizing pulse elicits only one impulse and no subsequent depolarization is observed. Furthermore, by weak orthodromic stimulation a single impulse can also be evoked. It has been shown that the frequency of discharge to a long outward current pulse can be graded by current intensity (Fig. 6). If prolonged synaptic excitation were responsible for repetitive discharge one would expect the frequency and duration of the reflexly evoked repetitive discharge to be related to the magnitude and duration of the synaptic potential. Figure 9 illustrates an experiment designed to explore this relationship. In A the repetitive response to a volley in the superficial peroneal nerve is shown. In B the same volley was delivered when the cell was hyperpolarized to an extent sufficient to prevent initiation of discharge, and only a prolonged synaptic potential was observed. The recording site was considered to be in the soma of the interneurone. C shows a plot, on the same time scale, of the inverse of interval between discharges in the response train, together with a tracing of the synaptic potential recorded during hyperpolarization. The possibility exists that some of the synaptic potential observed during hyperpolarization might have been due to inhibitory synaptic potential causing depolarization if the membrane potential had been greater than the equilibrium potential for that process. On the other hand, the fairly close correspondence between duration and amplitude of synaptic potential and discharge frequency supports the idea that prolonged synaptic excitation is a major factor in causing repetitive discharge. The lack of a prolonged subnormality and the rather small degree of adaptation permit the interneurone to discharge repetitively to prolonged synaptic excitation. This type of experiment cannot, of course, distinguish between a prolonged transmitter action following individual presynaptic impulses (cf. McIntyre, Mark & Steiner, 1956), or a briefer transmitter action resulting from temporally dispersed presynaptic impulses.

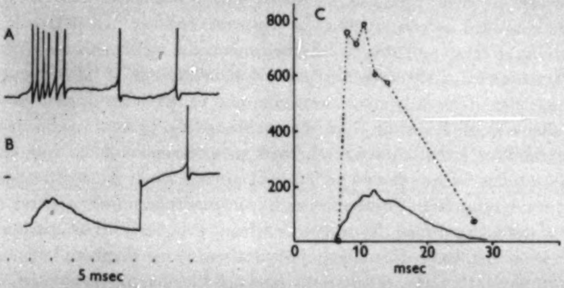

Fig. 9. Synaptic potential and repetitive discharge in an interneurone soma. A, response to a volley in deep peroneal nerve. B, repetitive response to same volley during passage of hyperpolarizing current; only synaptic potential evoked. C, plot of synaptic potential observed in B and inverse of interval between impulses in response train of A. Tracings from original records.

The latter, however, seems more probable, in view of the fact that weak afferent stimuli can evoke only a single impulse in an interneurone which responds by a repetitive train to stronger afferent stimuli. Furthermore, the 'spontaneous' synaptic potentials, presumably due to background impingement of presynaptic impulses as recorded by Haapanen *et al.* (1958), are of relatively brief duration.

DISCUSSION

Although interneurones in the spinal cord may not form a homogeneous group with respect to their properties, there are certain features which are common to units identified as interneurones in the present study. In particular, there are striking differences between interneurones and motoneurones. The latter cells have been more extensively studied, but behaviour of the type exhibited by spinal interneurones is much more common in the central nervous system. The difficulties of recording from interneurones by present techniques have been a limiting factor in studying their properties in detail but sufficient information has been obtained to indicate some of their general features.

Unit recordings from interneurones by intracellular micropipettes may be classified into two types, from axon or from cell body. The distinguishing feature is the presence of synaptic potentials of some magnitude (more than 1 mV) in the latter. In the present study KCl-filled micropipettes of high resistance were used. With such electrodes successful impalement of axons of primary afferent fibres and of motor axons occurs frequently; therefore a presumed recording site in interneurone axon is feasible. This identification is also suggested by the shorter average time constant from units considered to be interneurone axons than from interneurone somata, but the data are not conclusive. Unfortunately, other criteria for distinguishing axon from cell-body recording, such as are present in motoneurones (after-potential and double component spike) are lacking in interneurones. However, the assumption of different recording sites from interneurones is reasonable and avoids the necessity of postulating that some interneurone somata do not exhibit excitatory synaptic potentials (Haapanen *et al.* 1958). The same conclusions were implied in Frank & Fuortes's (1955) interpretation of potentials recorded from interneurones.

The time constant of interneurones was derived from measurement of strength–latency relations by direct stimulation. Synaptic potentials were considered too complex to permit estimation of time constant from their rate of decay. Further, such estimates assume displacement of membrane potential by brief synaptic action with subsequent passive decay. Evidence has been presented (Frank & Fuortes, 1956*b*; Eccles, 1957) indicating that the time constant measured by decay of synaptic potential is too long because of persistence of transmitter action. The use of high-resistance micropipettes which showed appreciable resistance changes on movement prevented the use of the time course of change in membrane potential on application or withdrawal of current for estimation of the time constant of interneurones. Frank & Fuortes (1956*b*) discussed similar difficulties in the use of this method in cat motoneurones, although it has been used successfully by Araki & Otani (1955) in toad motoneurones. In the present study, therefore, only one method of time constant estimation could be used. While the number of observations is limited, the time constant of interneurone somata does not differ greatly from that of motoneurone somata, although it may be slightly shorter. In contrast, the time constant of interneurone axons is similar to that in primary afferent fibres and in motoneurone axons.

Initiation of discharge in interneurones occurred at levels of excitatory synaptic potential of 2·5–10·9 mV (average 6·7 mV). From a limited number of observations this level does not appear to be significantly different from that in motoneurones (Coombs, Eccles & Fatt, 1955). The site of impulse initiation in motoneurones is considered by some authors to be the initial segment (Coombs, Curtis & Eccles, 1957). This view is suggested by the occurrence of an inflexion in the rising phase of the spike, considered to result from impulse initiation in the previous initial segment. In interneurones such inflexions have rarely been observed. If impulse initiation did occur in the axon rather than in the soma of such cells, there could be no appreciable delay in conduction at the axon hillock. In this connexion the results of Haapanen *et al.* (1958) are of interest for they show that the 'firing level' may vary greatly with changes in membrane potential brought about by natural stimulation.

One of the important characteristics of interneurones is the lack of prolonged hyperpolarization after discharge such as is found in motoneurones. This is undoubtedly a factor in determining the frequency at which these neurones can discharge. Subnormality limits the response frequency of motoneurones to about 50/sec, but interneurones can discharge at rates well in excess of 1000/sec. In order to respond at such frequencies, refractoriness following an impulse in interneurones must be nearly as brief as in A fibres.

Repetitive discharge in response to a single afferent volley is a common finding in unit recording from interneurones, the number of impulses depending on the size of the afferent volley. The response to direct depolarizing current pulses of sufficient duration and intensity is also repetitive, whereas brief current pulses can elicit a single impulse. The repetitive response to afferent volleys is due to long-lasting synaptic excitation. The part played by after-currents in the generation of repetitive discharge in spinal interneurones is certainly minimal, if present. The long synaptic excitation that occurs in many interneurones probably results from temporally dispersed presynaptic action. While no direct evidence has been obtained as to the duration of excitatory transmitter action in spinal interneurones, there is no reason to suggest this is particularly prolonged after impingement of a single presynaptic impulse. Other neurones within the central nervous system that show repetitive discharge may have different mechanisms involved. Some sensory relay neurones have excitatory presynaptic connexions that are probably less complex than the spinal interneurones studied. For example, cells of Clarke's column, certain thalamic relay cells, dorsal column relay cells in the gracilis and cuneate nuclei and neurones of the dorsal lateral geniculate may receive a direct presynaptic input without the interposition of other interneurones. Such relay neurones exhibit repetitive discharge following presynaptic stimulation that may depend upon long-lasting transmitter action or in some cases upon after-current mechanisms (McIntyre *et al.* 1956).

SUMMARY

1. The properties of spinal interneurones have been investigated in the acute spinal cat, using intracellular micropipettes. By means of a bridge circuit, stimulation was carried out through the same electrode used for recording.

2. Recordings from interneurones are considered to be from one of two sites, axon or soma. Those from the latter display conspicuous synaptic potentials. The time constants of interneurone somata and axons, motoneurone somata and axons, and primary afferent fibres are compared.

3. Excitatory synaptic potentials of interneurone somata elicited by nerve volleys are complex and appear to be compounded of temporally dispersed brief synaptic events. Impulses are initiated in association with synaptic potentials of 2·5–10·9 mV.

4. Spike potentials of interneurone somata are often followed by a brief post-spike positivity but lack the prolonged after-hyperpolarization characteristic of motoneurones.

5. Spike duration in interneurone somata does not differ appreciably from that in interneurone axons, but is briefer than that in motoneurones.

6. Interneurone somata show a much greater tendency to discharge repetitively to long depolarizing currents than do motoneurones. The frequency of discharge can be graded by current intensity. Some adaptation of interneurone response to constant current occurs.

7. Repetitive response of interneurones to orthodromic stimulation appears to result from prolonged suprathreshold synaptic excitation. Self re-excitation, by after-currents or by activity in recurrent pathways, appears to play a negligible part in repetitive discharge.

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Note added in proof. Since submission of this paper, Wall (1959, *J. Neurophysiol.* **22**, 305–320) has published a study on the repetitive discharge of neurones, including spinal interneurones. In the latter he noted the greater variation in later impulses of a repetitive response to nerve volleys, that reduction in volley size reduced the number of discharges and caused slight increase in latency and increased variation of the first response. Our findings confirm these observations. Wall also noted that direct stimulation by an intracellular electrode (presumably with a brief pulse) caused only one impulse to be initiated. He found that a direct stimulus interpolated before the response to a nerve volley could abolish the first response and alter the latency of the second but had no effect on the timing of subsequent impulses. He concluded that the origin of the discharge pattern must be external to the cell, an interpretation which gains support from the present study.

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BACKGROUND DISCHARGE AND EVOKED RESPONSES OF SPINAL INTERNEURONES

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The preceding paper (Hunt & Kuno, 1959) dealt with properties of spinal interneurones and mechanisms of synaptic excitation of such cells. The present study is concerned with an analysis of the background discharge of interneurones, the modulation of discharge by natural exteroceptive stimulation, and the response to nerve volleys from various peripheral sources. All these features of interneurone behaviour have been studied previously. Background discharge of interneurones has been noted by a number of authors (Frank & Fuortes 1955, 1956; Kolmodin 1957; Haapanen, Kolmodin & Skoglund 1958; Kolmodin & Skoglund 1958). Kolmodin (1957) has studied extensively the patterns of response of interneurones to muscle stretch and to a lesser extent to natural exteroceptive stimulation. Finally, several authors (Woodbury & Patton 1952; Frank & Fuortes 1955, 1956; Haapanen *et al.* 1958) have published some examples of the responses of interneurones to afferent volleys. The present study provides new information on these aspects of interneurone behaviour. The background discharge has been studied by an analysis of frequency distribution of impulse intervals, responses to natural exteroceptive stimulation have been subject to more extensive study, and a systematic study has been made of responses to nerve volleys from various peripheral sources. The interneurones that respond by high-frequency trains of impulses to antidromic volleys in ventral root fibres have been studied in detail (Renshaw, 1946; Eccles, Fatt & Koketsu 1954) and are not included in the present report.

METHODS

Intracellular recording was carried out in spinal cats by the methods described in the previous paper (Hunt & Kuno, 1959). For extracellular recording, glass micropipettes filled with indium were employed; these had tip diameters of 10–20μ and d.c. resistance of 8–12 kΩ. They were

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connected by a cathode follower to an a.c. preamplifier. Unless otherwise specified, nerve volleys were delivered at a rate of one every 2 sec. Other details were described in the previous communication. Over 270 spinal interneurones have been studied. The majority of units were recorded from segments L7 and S1, chiefly in dorsal horn. No histological control was carried out.

RESULTS

Background discharge

A large proportion of the interneurones examined exhibited background discharge. About 75% of the units studied by extracellular recording, and a slightly smaller percentage of those studied by intracellular recording, showed background discharge in the absence of added stimulation. Some examples of background discharge in interneurones are shown in Fig. 1. The upper record shows the potentials recorded intracellularly by a micropipette from a position judged to be in an interneurone soma. Impulses recurred at irregular intervals and were associated with progressive depolarizations before impulse initiation (prepotentials). In the intervals between impulses other fluctuations of membrane potential occurred. These subthreshold variations did not recur at regular intervals. The lower trace of Fig. 1 shows an intracellular recording from a position judged to be in an interneurone axon. In this case no appreciable prepotentials were seen before the 'spontaneous' impulses. The justification for considering recording sites as being from soma or axon was given in the preceding paper (Hunt & Kuno, 1959).

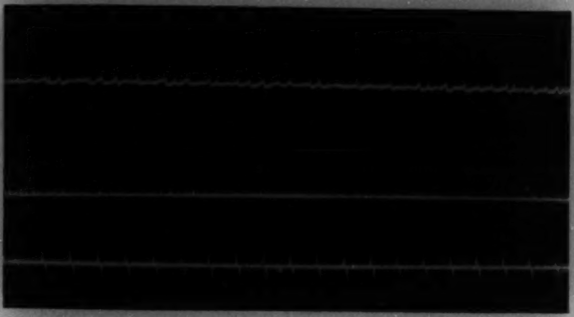

Fig. 1. Background discharge, intracellular records. Upper trace, from interneurone soma; note prepotentials and base-line fluctuation of membrane potential. Lower trace, from interneurone axon; no prepotentials were observed. Time marker 0·1 sec.

In a number of units continuous recordings of about 200–600 background discharges were made and each impulse interval was subsequently measured. A frequency distribution of impulse intervals was then constructed. Figure 2 shows some representative samples. In A the mean impulse interval was 35·6 msec and the total number of intervals counted was 391. The observed distribution in this case has been compared with the curve which indicates the expected distribution for random events given by the formula (Feller, 1950):

$$n = N\frac{\Delta t}{t}\exp\left[\frac{t}{T}\right],$$

where n is the number of occurrences of any interval between t and $t+\Delta t$, N is the total number of intervals, and T is the length of the mean interval. The frequency distribution of impulse intervals of this unit was distributed in a manner suggesting random discharge. Had sufficiently small class intervals

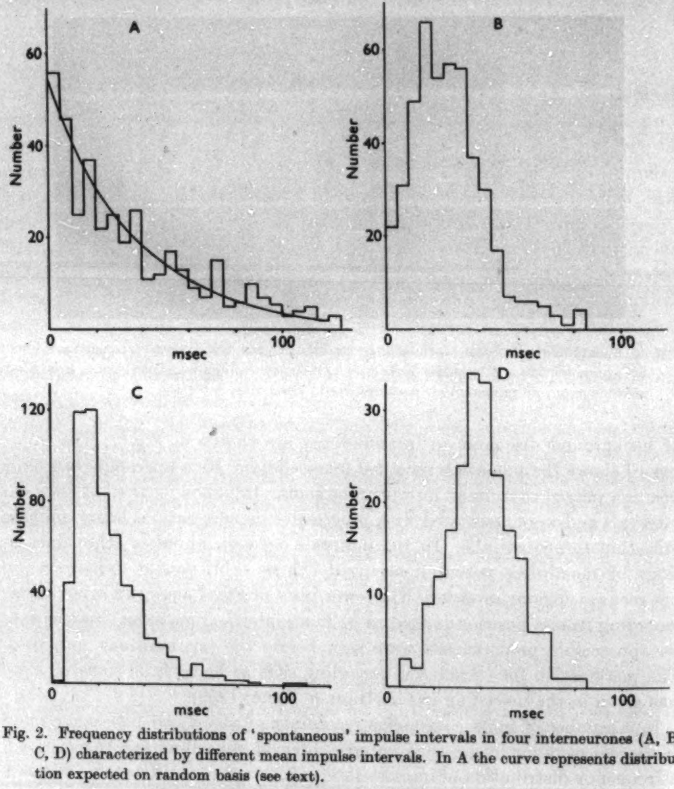

Fig. 2. Frequency distributions of 'spontaneous' impulse intervals in four interneurones (A, B, C, D) characterized by different mean impulse intervals. In A the curve represents distribution expected on random basis (see text).

been used, one would have expected a smaller number of occurrences for the least interval because of refractoriness.

The units of Fig. 2B, C and D have frequency distributions of impulse intervals which obviously depart from that expected with purely random discharge and which indicate a rhythmic generation of impulses subject to considerable fluctuation. The histogram of Fig. 2D shows a nearly normal distribution, while those of Figs. 2B and C show considerable skewness. When the mean interval was longer, the distribution was more nearly normal. These results may be interpreted as indicating a rhythmic excitation of interneurones subject to random fluctuation. A similar analysis of spontaneous discharge of Betz cells in cortex has been reported by Martin & Branch (1958). The present observations provide little direct evidence about the mechanism of generation of 'spontaneous' discharge; that is, whether it is caused by autorhythmic activity in individual cells or by excitation in networks of interneurones. In these acute spinal preparations there is a very considerable background of afferent activity entering the cord from various receptors. The large variation in impulse interval, even in interneurones exhibiting a tendency toward rhythmic discharge, suggests that the background impingement of synaptic influences on the interneurone plays an important part in the initiation of 'spontaneous' discharge.

Natural stimulation

Kolmodin (1957) has investigated in detail the responses of spinal interneurones to proprioceptive natural stimuli. The time course of frequency changes of interneurone discharge to muscle stretch has been investigated and the convergence patterns from various muscles, as well as from exteroceptive sources, has been described. The interaction between proprioceptive and exteroceptive natural stimulation on interneurones was also described. The emphasis in Kolmodin's study was on proprioceptive stimulation and responses to exteroceptive natural stimulation was not reported in detail. In the present study stimulation of skin and paw areas of the lower hind limb was carried out using light touch, bending of hairs, pressure, or pinching. By exploring the peripheral receptive field to which the natural stimulus was effective, the majority of units studied were found to be affected by stimulation of skin over a wide area. This might be expected from the large amount of convergence shown in response to nerve volleys (see p. 376). However, a number of interneurones were found which showed an increase in discharge only to stimulation of a discrete and small receptive field. Such units were usually excited specifically by bending of hairs in the receptive area and showed a high-frequency burst on bending the hairs and another burst on release, with little or no discharge when the hair was held in the bent position. The adaptation in this case may be attributed largely to the receptor, since a rapidly adapting discharge of this type is seen in afferent fibres from hair receptors.

The response of most interneurones to touch or pressure stimuli was maintained for the duration of the stimulus. Although it was not possible to be certain of constancy of stimulus intensity, there appeared to be relatively little adaptation in the interneurone response to maintained pressure or touch (Fig. 3). Kolmodin (1957) also noted, in studying the interaction between proprioceptive stimulation and pressure to the pad of the foot, that the effect from the latter showed little adaptation. Slowly adapting receptors responding to touch were noted in the foot pad by Adrian & Umrath (1929).

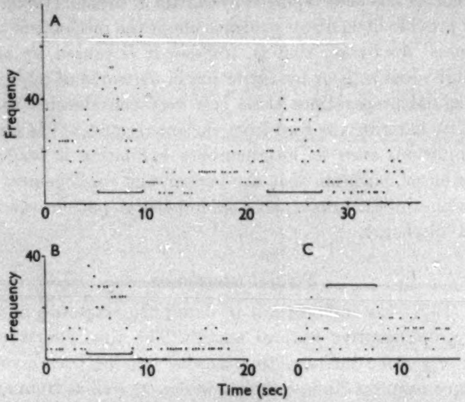

Fig. 3. Frequency changes in discharge of a spinal neurone to (A) pressure first to ipsilateral paw then to contralateral paw, (B) squeezing skin over tendo Achillis (ipsilateral) and (C) touch of skin over trochanter of ipsilateral femur. In this and subsequent records bar indicates approximate duration of stimulus.

A variety of patterns of convergence of excitatory and inhibitory influences from different peripheral sources was observed. One type pattern is shown in Fig. 3. Impulse intervals were measured from continuous photographic records and the inverse of interval (or frequency) calculated. Fig. 3A shows the increase in discharge frequency that followed pressure to the ipsilateral paw and then to the contralateral paw. Fig. 3B illustrates the increase in discharge that accompanied squeezing the skin over the ipsilateral tendo Achillis, and Fig. 3 C shows the inhibition of spontaneous discharge that occurred with touch of the skin over the trochanter of the ipsilateral femur. A different pattern was found in the unit of Fig. 4. In this case pressure to the ipsilateral paw caused an inhibition of base-line discharge maintained for the duration of stimulation (A), but similar stimulation of the contralateral paw increased the discharge frequency (B). The converse of this pattern was also observed.

The responses of 75 interneurones to stimulation of ipsilateral or contralateral paw have been tabulated in Table 1. The patterns most frequently observed were excitation from ipsilateral paw and inhibition from contralateral paw, or excitation from ipsilateral paw and no effect from contralateral paw. Excitation from both paws was fairly common. Inhibition from ipsilateral paw and contralateral excitation was not infrequent. No effect from either paw was seen in eleven units. In general, ipsilateral inhibitory effects were usually associated with demonstrable contralateral effects, but ipsilateral excitatory action could often be found with no evident crossed effect. Frank & Fuortes (1956) have also described an interneurone with excitation by contralateral skin stimulation and inhibition by ipsilateral skin stimulation, as well as one which was excited by both.

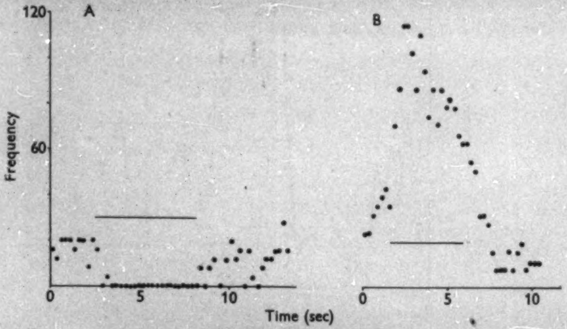

Fig. 4. Frequency changes in discharge of an interneurone to (A) pressure to ipsilateral paw and (B) pressure to contralateral paw.

TABLE 1. Effects of paw stimulation

Number of units	Contralateral paw	Ipsilateral paw
9	+	+
22	−	+
6	+	−
4	−	−
21	0	+
11	0	0
1	+	0
1	−	0
0	0	−

Responses to natural stimulation sometimes showed a mixture of excitatory and inhibitory effects. In the unit of Fig. 5, pressure to the ipsilateral paw caused initial inhibition of discharge followed by increase in frequency above the base-line level. The interneurone of Fig. 6, on the other hand, responded to pressure to the ipsilateral hind paw by an initial acceleration of discharge leading to a marked inhibition (A). This unit is also of interest in that an excitatory effect of ipsilateral forelimb stimulation could readily be shown (B).

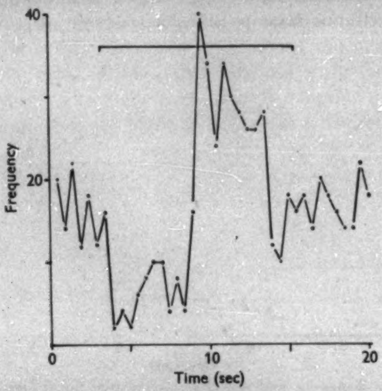

Fig. 5. Frequency changes in discharge of a spinal interneurone to pressure to ipsilateral paw.

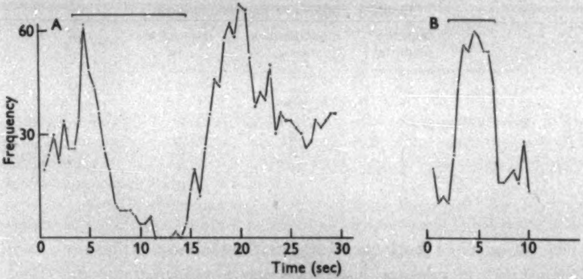

Fig. 6. Frequency changes in discharge of a spinal interneurone to (A) pressure to ipsilateral hind paw and (B) squeezing ipsilateral forelimb.

A few units showed clear evidence of having a discrete receptive field causing excitation while stimulation of nearby areas produced inhibition. One such interneurone (Fig. 7) had an increase in discharge frequency in response to touching the 5th digit of the ipsilateral hind paw (B) yet touch of the 2nd digit produced inhibition of the base-line discharge (A). This pattern bears some similarity to lateral inhibition in the *Limulus* eye (Hartline, Wagner & MacNichol, 1952), on units with ‘off’ surround in the cat eye (Kuffler, 1952), and to response of cortical neurones to natural stimulation in which there is an excitatory peripheral field with an inhibitory surround (Mountcastle, 1957).

The discharge frequency of an interneurone could be graded by intensity of natural stimulation. Maximal frequencies varied widely among different units: in some, natural stimulation caused discharge frequency to rise above 250/sec (cf. Frank & Fuortes, 1956). In general, excitatory responses to natural stimuli were at lower frequencies than those to nerve volleys (see below).

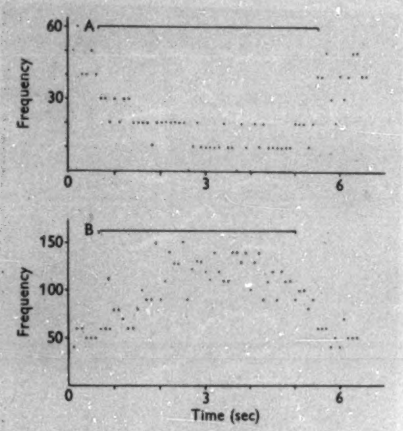

Fig. 7. Frequency changes in discharge of a spinal interneurone to (A) touch of 2nd digit and (B) touch of 5th digit of ipsilateral paw.

Responses to nerve volleys

As has been noted above, the response of a spinal interneurone to a single volley in a peripheral nerve usually consists of a repetitive train of impulses. The number and frequency of impulses in the response train varies among different units and with the size and source of the afferent volley. In spite of the variation seen, certain general features of the response pattern can be typified, with a caution that there is no clear-cut delineation between categories.

General features of interneurone discharge. Figure 8 illustrates the response of an interneurone to volleys of graded size in the plantar nerve. The number of discharges can be seen to increase with afferent volley size, the latter being recorded at the root-cord junction. Also, as the volley size was increased the latency of the first impulse was shortened and the frequency of discharge during the response train increased. In order to present these findings in a manner that permits easier comparison a graphic representation has been used. Each impulse was plotted as a point on the graph, the abscissa indicating latency from stimulus (or from volley entry at cord) and the ordinate the reciprocal of the interval between the impulse being plotted and the preceding one. The data of Fig. 8 are presented in this way in Fig. 9, latencies being plotted from the time of arrival of the most rapidly conducting afferent impulses at the root-cord junction. At stimulus strength 1·00, two impulses were evoked, the latency of the first being 1·4 msec. At strength 1·45, two impulses again were evoked but the latency of the first was briefer and the impulse interval shorter. At strength 2·24, four impulses were evoked, the first two being nearly identical in latency with those at strength 1·45. At stimulus strength 2·56, six impulses were evoked, the highest frequency being attained between the third and fourth impulses. In the latter case the response was not of greater duration than at strength 2·24, but was of higher frequency.

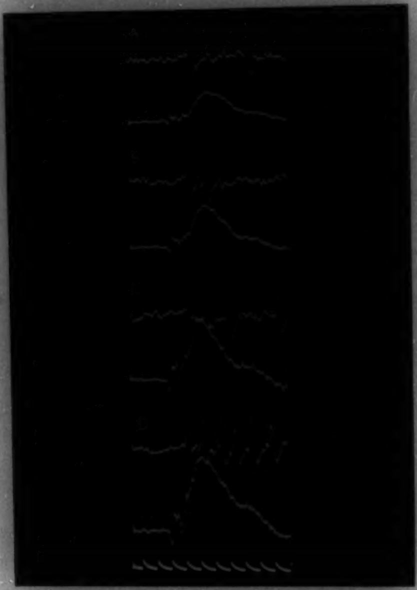

Fig. 8. Interneurone response to graded volleys in plantar nerve. Stimulus strength increased from A to D. Each record shows, on upper trace, recording of interneurone response by indium electrode; on lower trace potential recorded from lead at root-cord junction. Time marker, 1 msec. (Spikes retouched.)

Input-output studies were carried out on a number of interneurones that responded to sural volleys. Input size was measured by monophasic recording of spike potential amplitude in the distal cut end of the sural nerve. At the conduction distance used, the initial component of the compound spike potential was produced by fibres conducting at 30–70 msec. The number of discharges in the response train of interneurones could be finely graded by size of this initial component. Further, in the units examined input had to exceed 10% of the initial spike amplitude before interneurone discharge resulted. This suggests that summation is required for initiation of discharge in the neurones examined. Increase in stimulus strength, to bring in more slowly-conducting sural afferent fibres, often further increased the number of impulses in the response train. In view of the limited sample it is not possible to generalize as to the need for summation in interneurones.

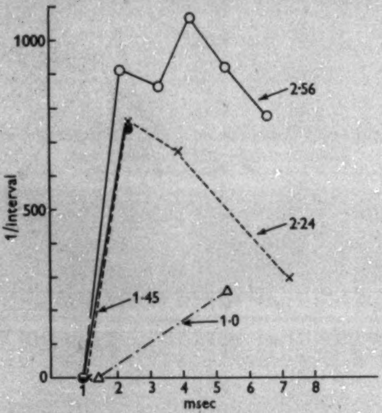

Fig. 9. Inverse interval–latency relation of responses shown in Fig. 8. Each impulse plotted on abscissa as latency after arrival of fastest component of plantar volley at cord, on ordinate as interval from preceding impulse. Stimulus strength in arbitrary units. See text.

Figure 10 shows the response of an interneurone to superficial peroneal volleys on three successive trials, the number of impulses evoked varying from five to seven. While the latencies of the first two impulses were quite constant, subsequent impulse intervals showed considerable fluctuation. The interneurone of Fig. 10 showed the highest discharge frequency after the third impulse in the discharge train. In this regard it differs not only from some other spinal interneurones but also from certain sensory relay neurones, which often show the highest frequency between the first and second impulse. In the experiment shown in Fig. 10, the arrival of the superficial peroneal

Figure 12 illustrates the response of an interneurone to afferent stimulation of a strength judged sufficient to excite C fibres. Weaker shocks failed to elicit any response. The long latency can be attributed in large part to afferent conduction time. This latency can be seen in A to amount to 214 msec. Record B shows a similar response recorded at a slower sweep speed. The duration of discharge was in excess of 450 msec. It may be noted in B that the frequency of discharge increased to a higher level some 200 msec after the onset of the response train. This unit also showed an occasional irregular spontaneous discharge and the only natural stimulus capable of eliciting discharge was very strong pinching of the ipsilateral paw.

Convergence. In many experiments stimulating electrodes were placed on the following peripheral nerves: biceps-semitendinosus (BST) triceps surae (TS), deep peroneal (DP), superficial peroneal (SP), plantar (P), and sural (S). Interneurones were then examined for discharge to volleys in each of these nerves. Table 2 presents a summary of the response patterns in 74 units for which complete data are available for all six volleys. The majority of the units examined showed responses to stimulation of more than one nerve. Thus 56 of the 74 interneurones were excited by volleys in three or more of the nerves tested. This indicates the large amount of convergence on many interneurones.

TABLE 2. Patterns of convergence on spinal interneurones

Number of units	Volley					
	BST	TS	DP	SP	S	P
12	+	+	+	+	+	+
12	0	+	+	+	+	+
13	0	0	+	+	+	+
8	0	0	0	+	+	+
7	0	0	0	0	+	+
3	0	0	0	0	0	+
4	0	0	0	+	0	+
2	0	0	+	0	0	+
8	0	0	+	+	0	+
2	0	+	0	0	0	+
1	0	0	0	0	+	0
1	0	0	+	0	0	0
1	0	+	0	+	0	+

For explanation of letters, see text.

Of the nerves tested, volleys in the plantar nerve excited the largest number of units. Sural and superficial peroneal nerve volleys also evoked discharge in a large number of units. In general, the muscle nerves examined excited a smaller number of interneurones. These findings indicate that among the interneurones examined in segment L7 and S1, largely from dorsal horn, the preponderance received excitatory connexions from nerves containing fibres from the paw and skin of the foot.

The temporal relation of discharge of interneurones to volleys from different sources was studied in several units. The latency of the initial impulse of the response train, the number of impulses and their frequency varied in a given interneurone with the source of the afferent fibres stimulated. Discharge due to volleys from different afferent sources was initiated with nearly the same latency in some interneurones. In others, latency varied widely with source of afferent volley. One factor determining these differences must be the complexity of neuronal connexions utilized in the excitatory pathway.

Inhibition. Inhibitory effects of afferent volleys on interneurones were indicated by reduction or cessation of spontaneous discharge or by inhibition of response to an excitatory afferent volley. As was noted by Frank & Fuortes (1956), inhibition sometimes followed discharge to nerve volleys, but inhibition without preceding discharge has also been frequently observed. Figure 13 illustrates the inhibition of response to an excitatory nerve volley. In A, a volley in the contralateral plantar nerve evoked a repetitive response of an interneurone. However, a conditioning volley in the ipsilateral plantar nerve at an appropriate interval beforehand (24 msec) inhibited this response (B).

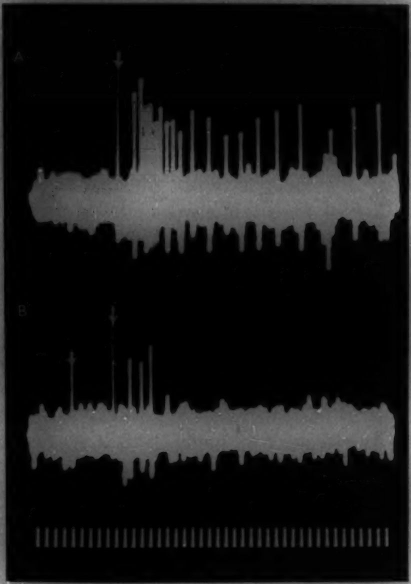

Fig. 13. Inhibition, by conditioning volley in ipsilateral plantar nerve, of the response of an interneurone to a volley in contralateral plantar nerve. A, contralateral plantar volley alone; B, ipsilateral plantar volley initiated 24 msec before stimulation of contralateral plantar volley. Arrows mark shock artifacts. Time marker, 5 msec.

The conditioning volley alone evoked no discharge. The duration of such inhibitory effects was studied in a number of interneurones. In general, as the conditioning–test interval was lengthened, the reduction in the number of responses to the test volley diminished. In Fig. 14A the inhibitory effect of a conditioning volley (plantar) can be seen as a decrease in the number of responses initiated by a test volley (superficial peroneal) here expressed as the

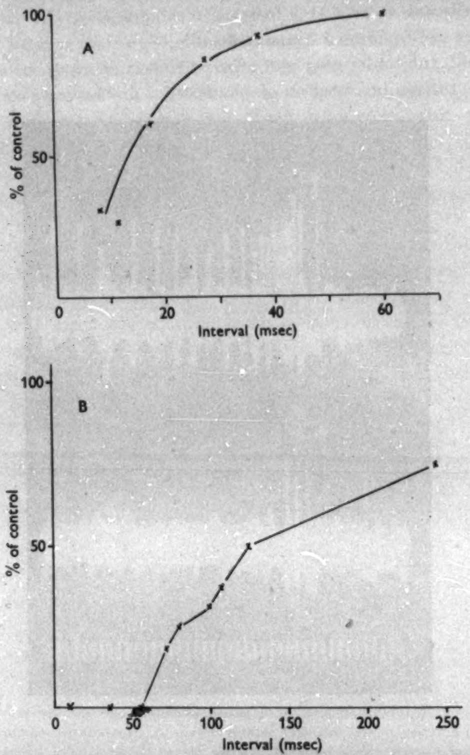

Fig. 14. Time course of inhibition. Ordinates, number of impulses fired by interneurone, expressed as percentage of number of impulses in control responses. Abscissae, interval between inhibitory and excitatory volleys. A, inhibitory volley in plantar nerve; testing excitatory volley in superficial peroneal nerve. B, inhibitory volley in sural nerve; testing volley in contralateral plantar nerve. A and B are from different interneurones.

percentage of control response. The inhibition decreased as the conditioning–test interval was lengthened, the total duration of the inhibition being about 60 msec. In Fig. 14 B the inhibitory effect of a sural volley on the test response to a contralateral plantar volley is shown for another interneurone. In this case the inhibition had a longer duration, in excess of 250 msec. From these observations it is clear that the number of responses to an excitatory volley can be finely graded by the degree of inhibition.

Volleys often showed a mixed effect on interneurones, usually discharge followed by inhibition. Activation of afferent fibres with dissimilar central effects probably accounts for some of these mixed actions and would also explain the finding of Frank & Fuortes (1956) that variation in frequency of afferent stimulation can alter the response from excitation to inhibition. This may be expected to occur when excitatory and inhibitory actions have different time courses.

Background discharge of a few interneurones was inhibited by ventral root stimulation. The same units discharged in response to volleys in peripheral nerves. The majority of interneurones examined showed no effect of antidromic ventral root volleys. However, a number of Renshaw cells were encountered when recording from the ventral horn; these showed the characteristic responses already described by Eccles *et al.* (1954) and Frank & Fuortes (1956).

Responses to repetitive stimulation. As has been recorded above and by other authors, the response of an interneurone to a nerve volley repeated infrequently often consists of a train of impulses. When the rate of repetition is increased above about 2–5/sec the earliest impulse continues to be evoked with little or no change in latency, but later impulses of the response train show greater lability and, with high rates of repetition, fail to appear. In many units the first impulse followed rates of stimulation in excess of 500/sec and in some cases over 1000/sec. To a tetanus of brief and constant duration to a peripheral nerve the number of impulses elicited in an interneurone was often maximal when the stimulus frequency was in the range of 50–100/sec. Although such units followed higher frequencies of stimulation, the number of responses per stimulus decreased. While interneurones varied with respect to the frequency of afferent stimulation that they could follow, the majority showed a much higher frequency limit than do motoneurones. The lack of a prolonged after-hyperpolarization and of consequent subnormality in interneurones as compared with motoneurones must be an important factor determining this difference (Hunt & Kuno, 1959).

DISCUSSION

The majority of the interneurones examined in the present study exhibited background discharge. This discharge was generally irregular but analysis of the frequency distribution of response intervals indicates, in most units, a rhythmic generation of impulses subject to considerable random fluctuation. Kolmodin & Skoglund (1958) recorded the fluctuations in membrane potential of motoneurones and interneurones that occurred 'spontaneously' or in response to natural stimulation. They observed in some motoneurones and interneurones slow oscillations in membrane potential, some of which were not associated with impulse initiation. They suggested that the rhythmic discharge in such neurones is primarily determined by a slow-oscillation tendency intrinsic to the membrane. In the present study oscillatory fluctuations of membrane potential in interneurones have not been found to be regularly recurrent. Furthermore, the background discharge usually displayed impulse intervals that were irregular. Analysis of the frequency distribution of impulse intervals indicates that even in units showing some tendency to rhythmic generation of impulses there is considerable fluctuation of impulse interval. These factors do not exclude inherent rhythmicity of the interneurone membrane as a factor in the generation of rhythmic discharge. They do suggest however, that the background impingement of excitatory and inhibitory effects from presynaptic bombardment plays an important part. A number of interneurones have shown bursts of discharge followed by periods of lower frequency or of silence, but the latter periods have not usually recurred at a regular rate. Regularly recurring cycles of modulation of interneurone discharge frequency, as reported by Frank & Fuortes (1956) in decerebrate cats, have rarely been seen. The difference is probably due to a greater preservation of integrated mechanisms in the decerebrate as compared with the acute spinal preparation. It is also possible that background discharge of spinal interneurones, in preparations in which supraspinal influences are still present, might show less random variation than in the acute spinal cat. No analyses of frequency distribution of response intervals have yet been made in the former.

Tonic activity in interneurones must influence the background excitability of other interneurones and of motoneurones and hence the capacity of the latter to respond in reflex acts. In the majority of interneurones the effect of natural stimulation is to increase or decrease the discharge frequency. In general, this modulation of frequency of interneurone discharge by activity in primary afferent fibres from exteroceptive sources appears to occur with little adaptation on the part of the interneurone. This appears also to be true in the case of most interneurones excited by proprioceptive input (Kolmodin, 1957). These findings are in keeping with the observation already reported (Hunt & Kuno, 1959) that little adaptation occurs in the response of interneurones to direct current.

A variety of patterns of response to stimulation of different peripheral sources has been noted among the interneurones examined. This is to be expected from the fact that analyses thus far have dealt with rather heterogeneous collections of interneurones. Since it has not yet been possible to determine the consequences of activity in a particular interneurone it is difficult to relate its behaviour to various types of natural stimulation with its role in reflex function. From knowledge of motoneurone responses and polysynaptic reflexes it is possible to surmise what pattern of interneurone behaviour would be appropriate. Some of the interneurones examined may be concerned with ascending systems; for example, certain units which responded only to bending of hairs in a small area could be concerned with transmission of information relating to location of sensation. The possibility should be kept in mind that the same interneurones may be concerned both with segmental spinal reflexes and ascending systems.

A conspicuous feature of many interneurones is the repetitive response to nerve volleys. The response train evoked by a volley in a peripheral nerve usually attains its peak frequency after the second or later impulses in the train. In contrast, certain sensory relay neurones, which also respond repetitively to incoming volleys, appear most commonly to attain the maximum frequency of response between the first and second impulses of the response train (Rose & Mountcastle, 1954; Laporte, Lundberg & Oscarsson, 1956). The fact that the maximal frequency in the response train of spinal interneurones can occur comparatively late suggests that the intensity of synaptic activation does not reach its maximum until that time. In contrast, the repetitive discharge of at least some sensory relay neurones may depend upon a synaptic excitation which reaches its peak early and declines smoothly thereafter. In sensory relay neurones, such as those of Clarke's column, repetitive discharge may follow a presynaptic input which is considerably more synchronous than that which impinges upon spinal interneurones (Lloyd & McIntyre, 1950; Laporte *et al.* 1956). In such cases the production of repetitive discharge may depend upon a prolonged synaptic excitation due to sustained transmitter action (McIntyre, Mark & Steiner, 1956). As was shown in the previous paper (Hunt & Kuno, 1959), the interneurones now studied exhibit synaptic potentials which appeared to be compounded from temporally dispersed presynaptic impingement, presumably resulting from activation of neuronal networks of varying complexity. The patterns of response described in the present study support this view. It has also been found that the later impulses of a repetitive response train to a peripheral nerve volley show considerably more variation in latency than do the earlier impulses of the train. This difference probably depends upon the fact that the later impulses of the train result from excitation mediated through more complex connexions of the interneurones. The greater lability would therefore be compounded of variability and a number of interneurone junctions serially excited.

The fact of repetitive discharge may be an important factor in the prolongation of activity in chains of interneurones. For example, if two cells, *A* and *B*, are connected in series, synaptic excitation of *A* for a given period will result in a train of impulses while the excitation is suprathreshold. This train of impulses may be expected to exert a more prolonged synaptic excitation of *B*, in turn generating a longer train of impulses. In this manner prolonged activation of interneurones might occur through a relatively short chain of cells connected in series.

It is common practice to assign a value for synaptic delay and from this to estimate the number of interneurones serially excited in a reflex response from the latency of the reflex less allowances for conduction. However, in the case of a motoneurone caused to discharge in a polysynaptic reflex the repetitive response of interneurones must be considered. The discharge frequency of an interneurone often reaches its maximum between the second and third impulses of its repetitive response train, sometimes later. By temporal summation one may expect the synaptic effect it exerts to reach a maximum some time later than the earliest impulse of the response train. Thus, in the case of a three-neurone reflex arc motoneurone discharge may occur considerably later than would be estimated by the number of synaptic delays. Some earlier experiments of Lloyd (1943) may be pertinent to this argument. He noted that certain polysynaptic reflexes had a latency indicating transmission through an arc of three neurones only when powerfully facilitated. In the absence of added facilitation latency was longer. Presumably only in the facilitated state would the first impulse of the response train of the interneurone be effective in initiating motoneurone discharge. Clearly, in view of the frequent occurrence of the repetitive discharge in interneurones, calculations of the number of neurones intercalated in a reflex response must not be made from central latency simply on the basis of a fixed value of synaptic delay. The high frequency of interneurone discharge observed in many units suggests that temporal facilitation must play an important part in the transmission of polysynaptic reflexes evoked by single afferent volleys.

SUMMARY

1. A study has been made of the background discharge of spinal interneurones and their responses to natural stimulation and to nerve volleys in acute spinal cats.

2. Background discharge is characteristically irregular. While frequency distribution of impulse intervals may approach that expected from random probability when mean impulse interval is short, units with longer mean impulse intervals show a distribution indicating rhythmic generation of impulses subject to considerable random variation.

3. Intracellular recording from interneurone somata reveals fluctuation in

membrane potential and prepotentials associated with 'spontaneous' discharge. Such changes are absent in records from axons.

4. Interneurone discharge shows little adaptation to maintained natural stimuli except when activated from receptors known to adapt rapidly.

5. Some interneurones show mixed effects of excitation and inhibition from the same receptive field. Also, certain interneurones have an excitatory receptive field adjacent to an inhibitory one.

6. Repetitive responses to single afferent volleys often display maximal frequency after the second, third, or later impulses.

7. The number of impulses in a response train may be graded by the size of an afferent volley. Increasing strength of afferent stimulation decreases latency of the initial impulse of the response train to some minimum and increases frequency of discharge. Earlier impulses show less latency variation than later impulses of a response train and more readily follow afferent volleys delivered at high repetition rates.

8. Patterns of convergence of afferent volleys from different peripheral sources are described. A large fraction of the select sample of interneurones examined have shown excitatory effects on stimulation of fibres from cutaneous sources.

9. Features of inhibition of response of spinal interneurones by afferent volleys are described.

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FAST SUPRASPINAL CONTROL OF MAMMALIAN MUSCLE SPINDLES: EXTRA- AND INTRAFUSAL CO-ACTIVATION

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Histologists (Cilimbaris, 1910; Garven, 1925, Barker, 1948; Cooper & Daniel, 1956) have described motor fibres entering the polar regions of mammalian muscle spindles which appear to be larger in diameter than γ motor fibres (Leksell, 1945). In fact, a fibre which is thin just before forming an intrafusal motor ending may not necessarily be of small diameter throughout its length, since Eccles & Sherrington (1930) have demonstrated that larger motor fibres in muscle nerves give off smaller branches as they approach the muscle. Cooper & Daniel (1956) have expressed surprise that the problem of large-fibre motor innervation of mammalian spindles has been neglected in physiological work. This may be understood, in view of the complications which arise when a precise definition of the problem is attempted.

The motor fibres concerned are liable to stimulate intrafusal muscle at the very moment when extrafusal contraction begins. Consider now the situation of the spindles as they lie in parallel with the extrafusal muscle bundles. Proximally spindles are inserted on the endomysium or perimysium of extrafusal fibres. Distally, many spindles taper out into special tendinous slips inserted on aponeuroses or tendons, but those within the belly of the muscle usually stop short, to be inserted again on extrafusal endo- or perimysium (Sherrington, 1894; Huber & de Witt, 1897; Hinsey, 1927; Barker, 1948). In a twitch, therefore, one or both points of insertion will move with the extrafusal muscle as it shortens. Hence the effectiveness of the α fibre spindle innervation in terms of sensory discharge will depend upon the relative velocities of shortening of spindle fibres receiving such innervation and the extrafusal fibres to which they are attached, as well as upon the relative conduction velocities of intra- and extrafusal motor axons within the α group. No data of this kind are available. As a further complication, it may be that the large fibres seen entering mammalian spindles are branches of ordinary α fibres destined for extrafusal elements (Cooper & Daniel, 1956).

In a study of unit spindle afferents in dorsal root filaments, Hunt & Kuffler (1951) described an early discharge occurring when muscle contractions were set up by stimuli to motor nerves or ventral roots, but concluded that this was the result of tension changes in extrafusal muscle rather than a specific motor innervation of the spindles themselves. We have repeated their experiments with additions of our own in the following paper (Granit, Pompeiano & Waltman, 1959), but at the moment we propose something of a short-cut through the complications outlined above. We shall describe the effect of stimulating some regions of the brain stem which project to the spinal cord and raise the simple question of whether, under such circumstances, spindles discharge early enough to be activated by α fibres. This is a short-cut because it presents a physiological problem which is, in the first instance, independent of the peripheral mechanism by which such activation takes place. If spindles are co-activated in small contractions set up by, say, pyramidal stimulation, then this co-activation is likely to be a physiological mechanism in its own right, independently of whether it is the result of mechanical or nervous integration within the muscle.

METHODS

The technique of the present study has been used in previous publications from this laboratory (Granit & Kaada, 1952; Eldred, Granit & Merton, 1953; Granit, 1958). Spindle afferents arising in ankle muscles were functionally isolated from dorsal roots of twenty-four cats which had been given 20 mg chloralose and 10 mg pentobarbitone per kilogram, or else were decerebrated under ether. The former animals sometimes received additional small doses of pentobarbitone as the experiment progressed. In several animals records were also taken from filaments of ipsilateral ventral roots L7 and S1. The hind limb was denervated except for the branches of the tibial nerve to gastrocnemius, plantaris and soleus when these ankle extensors were used, or the appropriate branches of the peroneal nerve when the physiological ankle flexors, tibialis anterior and extensor digitorum longus, were used. Stimulating electrodes were placed on the tibial or peroneal nerve at knee level so that spindle afferents could be identified by their pause in a motor twitch as well as by their response to stretch and pinna twist (the γ reflex of Granit, Job & Kaada, 1952), and so that afferent conduction times from knee to dorsal root could be measured. The limb was rigidly fixed at hip, knee and ankle, and the appropriate muscle was connected to an isometric strain-gauge myograph or else left unhooked. The term 'slack muscle' refers to the latter condition, while zero extension is that length which just produces a deflexion on the myograph of 10 g. For supraspinal stimulation, a stainless steel needle, 0·5 mm in diameter and insulated to the tip, was placed in the brain by stereotaxic methods, and square pulses were applied between it and a large electrode on the divided temporal muscle. At the end of an experiment, electrolytic lesions were made at each stimulus site and then identified in serial paraffin sections stained alternately by Nissl's and Weil's methods.

RESULTS

Time of γ loop. Knowledge of this time is a necessary preliminary to the present work. From records published by Kuffler, Hunt & Quilliam (1951) and Hunt (1951) on stimulation of γ fibres in ventral roots, loop times of the order of 12–15 msec can be derived, counting from ventral to dorsal root.

Granit & Holmgren (1955(observed latencies varying from 10 to 20 msec, but at the time the possibility of large-fibre motor innervation of spindles was not considered. Accurate estimates cannot always be obtained since many spindles are not properly driven by stimulation of ventral root or nerve; instead, the afferent discharge slowly waxes and wanes and the latent period, if at all discernible, may be anywhere between 15 and 30 msec.

In order to exclude the effects of extrafusal contraction when measuring the latency of the γ loop, use was made of the drug Flaxedil (gallamine triethiodide; May and Baker Ltd.), which in mammals fully paralyses α motor end-plates long before the γ system is silenced (Granit, Homma & Matthews, 1959). Figure 1 shows records obtained from a flexor spindle afferent which had strong γ control, as judged by the precision with which it was driven by nerve shocks as well as by a flexor reflex. Uppermost are controls at both slow and fast speed with three motor twitches to illustrate the regularity of the spindle's response. P is a reflex evoked by a stimulus to the central end of the cut ipsilateral popliteal nerve; this excites the spindle without causing a detectable

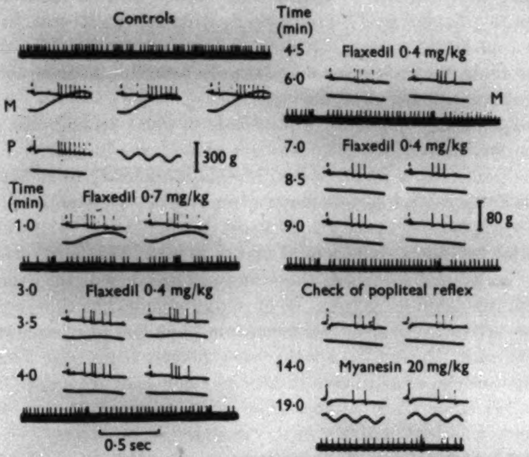

Fig. 1. Effect of Flaxedil on discharge of spindle afferent from extensor digitorum longus. Simultaneous records at slow speed sometimes added to sweeps run with time 100 c/s, as marked (left, middle). Slow speed, as marked below (left) for 0·5 sec. M, myogram. Stimulating muscle nerve at knee level with 0·5 V, 0·5 msec pulses except in P which shows flexor reflex excitation of popliteal nerve, checked later in the experiment. Flaxedil injected in doses and at times indicated, followed by tests of effect from nerve. Experiment ends with injection of Myanesin. Note change of sensitivity of myograph from 300 g to 80 g calibration made at time 9·0 min.

contraction at the given myograph sensitivity. Now the loop time for the earliest spike, about 15 msec, might include some delay as a result of extrafusal shortening making the spindle slack. To remove the contraction, Flaxedil was administered in small doses, while myograph sensitivity was augmented. There was a minimum latency of 10 msec, lengthening to 12 msec as more Flaxedil was given. The effect of the drug is also seen in the diminution and regularization of the resting discharge, but the direct response to nerve stimuli as well as the popliteal γ reflex remained to the very end, when extrafusal contraction was gone and γ bias considerably reduced. That some γ bias may have been left is suggested but not proved by the last test with Myanesin (3-ortho-toloxy-1,2-propanediol; British Drug Houses, Ltd.), which removes supraspinal γ control (Granit & Holmgren, 1955). The further decrease in discharge frequency may also have been due to some reduction in blood pressure consequent on the injection.

This experiment represents an ideal case of good driving. The minimum loop time, from nerve at knee level to dorsal root, was 10 msec. To obtain the loop time from ventral root to dorsal root, it is necessary to add the conduction time from ventral root to nerve at knee level, a distance of 15 cm. The average γ conduction velocity is 30 m/sec (Kuffler *et al.* 1951), which means the addition of 5 msec, giving a total time of 15 msec. This agrees well with the values of earlier studies quoted above and will be taken as the average γ loop time from ventral to dorsal root. It of course includes the time consumed by the γ fibre activation of intrafusal muscle and the subsequent excitation of the spindle afferent, apparently a rather slow process since only 2 msec of the total loop time can be attributed to afferent conduction from spindle to dorsal root. Boyd's (1959) observation of slow intrafusal contraction in isolated cat tenuissimus muscle is pertinent in this regard.

Supraspinal stimulation. Several descending pathways may be stimulated from the brain stem in order to excite motoneurones innervating hind-limb muscles. Of them the pyramidal tract is the best known from both anatomical and physiological studies. Although this tract in the cat contains chiefly small and hence slowly conducting fibres, there are larger fibres in it which descend to the lumbar and sacral spinal segments (for a summary, see Lassek, 1954). These fibres connect with motoneurones indirectly, via interneurones which lie in the zona intermedia at the base of the ventral horn (Szentagothai-Schimert, 1941).

Lloyd (1941) has shown that when the contralateral medullary pyramid is stimulated, descending impulses arrive in these internuncial pools with a latency of 4·5 msec, and after a further delay of as long as 7·5 msec, the motoneurones of the same segment are facilitated. However, Lloyd could reduce the internuncial delay to 1 msec by repetitive stimulation, so that the latency of the motoneurone facilitation became at the minimum 5·5 msec. In the present experiments the contralateral pyramid was stimulated at the level of the medullary olivary complex or of the facial nerve, and it was found that single rectangular shocks of 2–3·5 msec duration, given once a second, were optimal for exciting the ankle muscles and their spindles. The latencies of muscle contraction or spindle response, measured from the onset of the shock in all cases, as in the work of Granit & Holmgren (1955), were not significantly reduced when tetani of briefer shocks were used instead. In a few experiments, where ventral root filaments were monitored, each pyramidal stimulus evoked a motoneurone discharge, often repetitive, which began with an average latency of 6 msec, although latencies as short as 4·5 msec were occasionally seen. This suggests that a prolonged single shock was equivalent in effect to a brief train of shorter stimuli (cf. Brookhart, 1952).

From the above it follows that if the average γ loop time is 15 msec and if γ motoneurones fire 6 msec after a pyramidal shock, then pyramidal stimulation may drive a spindle afferent over the γ loop with an average latency of 21 msec. Therefore, if a faster peripheral loop is to be demonstrated, latencies consistently below this figure must be found.

Figure 2 illustrates a typical experiment on pyramidal stimulation. The muscle, gastrocnemius, was studied in the slack condition and at extensions of 4 and 10 mm. In each case of A, four control sweeps and four with stimulation are shown. The contractions obtained, as in other cases of pyramidal stimulation of extensors, were quite small. It may be seen that the earliest spindle response appears early in contraction. In B histograms have been constructed for the latencies measured from a total of 68 sweep records, and the earliest significant values lie between 12 and 14 msec. These are obviously too short to be explained by the average time of the γ loop, In fact, if the stimulus had been given to the ventral root instead, these latencies would be short for that loop. When the muscle was extended to 4 and 10 mm, these same short latencies were obtained and, in addition, a later discharge appeared at 20 msec or more. Such a response may have been mediated over the γ loop.

To reduce the labour of measuring latencies from large numbers of single sweep records, photographic superposition, at a sweep repetition rate of 1/sec, was used in many experiments. Figure 3 is an example of such a case. The stimulus site was again the contralateral medullary pyramid (C) but this time the spindle was in a flexor, tibialis anterior, which responded to each shock with a sizeable contraction. It was, in fact, always easier to evoke contractions in the flexors when the pyramidal tract was stimulated. In the uppermost row are shown records formed by the superposition of 30 consecutive traces of resting discharge, at each of three muscle lengths. The spikes are randomly distributed. In the next row each superimposed sweep begins with a shock to the pyramid. Consistent driving was obtained at latencies of

13–16 msec, again too early for the average γ loop. Below follow single sweep records which emphasize the most characteristic feature of our findings, namely that the discharge begins at the foot of the contraction. In B the events are shown at slower speed and at lower myograph sensitivity; after the early discharge, the spindle is silent during contraction.

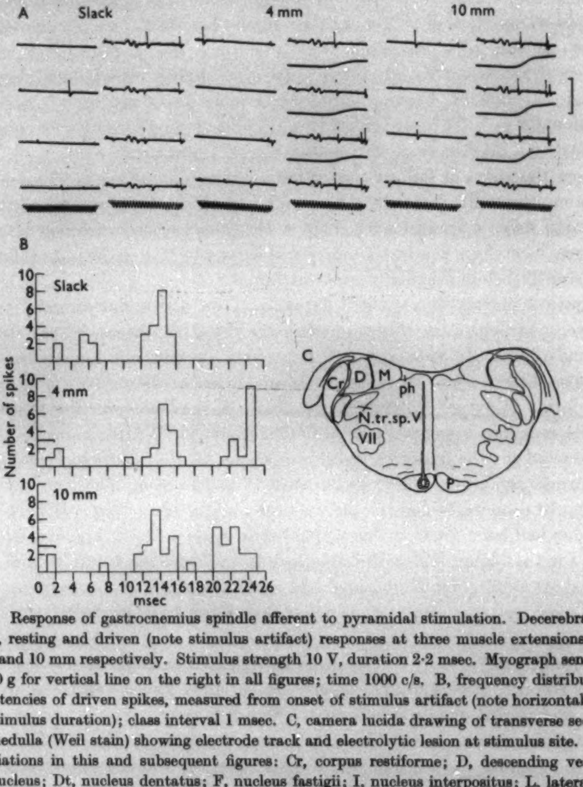

Fig. 2. Response of gastrocnemius spindle afferent to pyramidal stimulation. Decerebrate cat. A, resting and driven (note stimulus artifact) responses at three muscle extensions, slack, 4 and 10 mm respectively. Stimulus strength 10 V, duration 2·2 msec. Myograph sensitivity 80 g for vertical line on the right in all figures; time 1000 c/s. B, frequency distribution of latencies of driven spikes, measured from onset of stimulus artifact (note horizontal bar for stimulus duration); class interval 1 msec. C, camera lucida drawing of transverse section of medulla (Weil stain) showing electrode track and electrolytic lesion at stimulus site. Abbreviations in this and subsequent figures: Cr, corpus restiforme; D, descending vestibular nucleus; Dt, nucleus dentatus; F, nucleus fastigii; I, nucleus interpositus; L, lateral vestibular (Deiters's) nucleus; M, medial vestibular nucleus; N.tr.sp.V, nucleus tractus spinalis nervi V; P, pyramid; ph, nucleus praepositus hypoglossi; VII, motor nucleus of n. VII.

Although the latencies obtained with pyramidal stimulation were brief enough to argue against the γ loop, other sites were also tested for early spindle driving. Thus Fig. 4 illustrates an experiment in which the stimulus site was

Deiters's (lateral vestibular) nucleus of the ipsilateral side, the origin of the vestibulospinal tract.

Thulin (1953) stimulated Deiters's nucleus in the cat, using 5 msec pulses, and recorded the discharge in ventral roots L7 and S1; although latencies were not discussed, values of 4–5 msec from the *end* of the shock artifact may be estimated from his Fig. 2. In another study (Gernandt, Katsuki & Livingston, 1957) stimulation of the vestibular nerve with 0·5–1·0 msec shocks evoked a response in lumbosacral ventral roots with a minimum delay of 4–5 msec. Although these two reports are not quite in agreement with respect to latency, it does appear that in the cat the vestibulospinal tract, since it makes monosynaptic connexions with motoneurones (Schimert, 1938), may activate the motor horn somewhat sooner than the pyramidal tract. Recent anatomical observations (Pompeiano & Brodal, 1957) indicate that the spinal projection of Deiters's nucleus

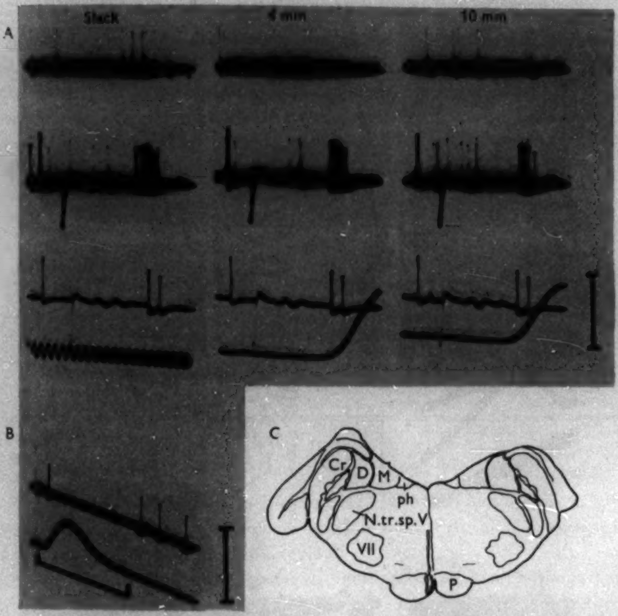

Fig. 3. Response of tibialis anterior spindle afferent to pyramidal stimulation. Decerebrate cat. A, upper and middle rows show resting and driven responses (30 superimposed sweeps each) at three muscle extensions, slack, 4 and 10 mm respectively. Lower row shows sample single sweeps with simultaneous myogram, calibration of myograph 80 g. Stimulus strength 20 V, duration 3·4 msec. Time 1000 c/s. B, single sweep record at slower speed (time 100 msec) and reduced myograph sensitivity 840 g. Note spindle pause after early discharge. Stimulus as in A. C, electrolytic lesion at stimulus site in contralateral pyramid.

is somatotopically organized, and that the dorsocaudal portion which was stimulated in this experiment (position 2 in C) projects to the lumbosacral cord and consequently to hind-limb muscles.

Only extensor muscles were studied during stimulation of Deiters's nucleus; in the experiment of Fig. 4 soleus was used and large contractions were obtained, as is seen in B. The uppermost records, formed by the superposition of 30 consecutive traces each, are controls showing the discharge at each of three muscle lengths; the lower records demonstrate the effects of stimulation. A response was consistently obtained with a latency as short as 13 msec. B illus-

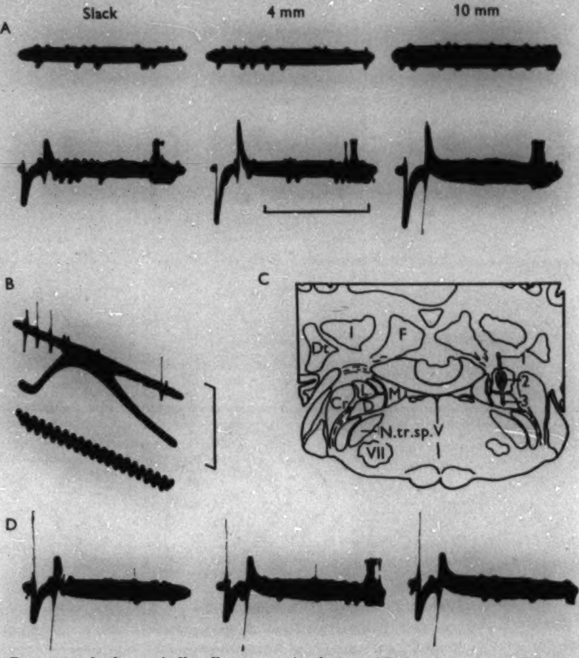

Fig. 4. Response of soleus spindle afferent to stimulation of Deiters's nucleus. Chloralose-nembutal cat. A, resting and driven responses (30 superimposed sweeps each) at three muscle extensions, slack, 4 and 10 mm respectively. Stimulus strength 5 V, duration 2·1 msec. Time 10 msec. B, single sweep record of driven response with myogram (calibration 300 g) at slower speed. Muscle at 4 mm, stimulus as in A, time 100 c/s. C, stimulus site, marked 2, in the hind-limb region of the ispilateral Deiters's nucleus. D, from left to right, responses obtained by stimulating at sites marked 1, 2 and 3 respectively, in C; muscle at 4 mm, stimulus 3·2 V, 2·1 msec, time as in A.

trates a contraction at slower sweep speed and two spikes coming at its foot. Figure 4 also demonstrates that the actual site of stimulation was quite well localized to the tip of the electrode, for in D it is clearly seen that positions 2 mm dorsal or ventral to that labelled '2' in C gave hardly any afferent response to threshold stimuli.

In other experiments, the medullary and pontine reticular formation (particularly the nuclei reticularis ventralis, gigantocellularis and pontocaudalis), the mesencephalic reticular formation, and the medial longitudinal bundle were stimulated, both ipsi- and contralaterally, yielding afferent responses with shortest latencies of 13, 14 and 11 msec, respectively. Spindle afferents from flexor and extensor muscles were studied. The reticular formation does not send fibres to the lumbosacral segments of the cord (Torvik & Brodal, 1957) but probably activates hind-limb motoneurones via propriospinal neurones, as suggested by Lloyd (1941). The medial longitudinal bundle was stimulated in its medial portion, which is said to contain descending fibres (see Nathan & Smith, 1955, for a review).

TABLE 1. Summary of earliest latencies of spindle responses to supraspinal stimulation: 60 observations on 45 spindles

Stimulus site	Earliest latency (msec)
Pyramid	12, 13, 13, 13, 14, 15, 16, 16, 16, 16, 16, 17, 17, 18, 18, 19, 20, 20, 21, 22, 23, 25. No response from 4 spindles
Medullary and pontine reticular formation	13, 15, 15, 15, 16, 16, 16, 17
Mesencephalic reticular formation	14, 15, 15, 17, 18, 19, 21, 25, 29, 30. No response from 4 spindles
Deiters's nucleus	13, 14, 15, 15, 15, 16, 19, 27. No response from 1 spindle
Medial longitudinal bundle	11, 14, 16

All observations on supraspinal stimulation have been summarized in Table 1. The total number exceeds the number of spindles because more than one stimulus site was often used in the study of a single spindle.

As a rule, spindles were driven with brief latencies from these selected sites only when measurable contractions were evoked, and the latency of the driven response in such a case was then found to bear a rather close relationship to the onset of the contraction. This was rather evident from the examination of sweep records but is more clearly shown by Fig. 5, which gives the distribution of time intervals between onset of contraction (zero time) and initiation of spindle response for a group of 31 observations on 20 spindle afferents. A high myograph sensitivity (80 g/cm film) permitted the measurement of time of onset of contraction to within ±1 msec, while the time of initiation of the spindle response was obtained in the following way. The afferent conduction time from knee electrodes to dorsal root (an average distance of 15 cm) was measured in each case and the afferent fibre conduction velocity calculated. The additional afferent time from muscle to knee was then estimated on the basis of average measured distances of 2 cm for gastrocnemius, 3 cm for plantaris, 4 cm for soleus, 6 cm for tibialis anterior and 8 cm for extensor digitorum longus. The total afferent conduction time from muscle to dorsal root so derived, although neglecting afferent time within the muscle itself, was considered adequate for the present purpose, and when subtracted from the latency of the driven spike, gave the initiation time. It is evident from the histogram that most responses began in the first 2 msec after the onset of contraction and that the total spread was quite narrow. Yet the absolute latencies of contraction and spindle response had wide ranges, 11–24 and 12–29 msec, respectively. This may be taken to indicate that the spindle discharge in each instance has been evoked by some mechanism operating with approximately the same speed as that of the fibres exciting extrafusal muscle, i.e. α fibres. Without the added evidence of the short latencies of many driven responses, this in itself suggests that the γ loop was not here involved.

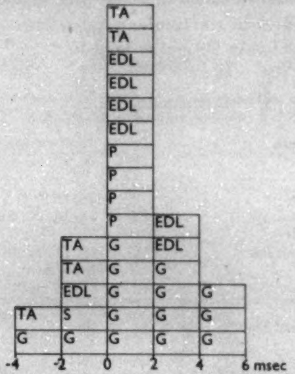

Fig. 5. Relation between time of initiation of spindle response and time of onset of contraction, in 31 cases of supraspinal stimulation. Time of initiation estimated by subtracting afferent conduction time from total latency, for each case. Abscissae represent differences between initiation time and time of onset of contraction, the latter set at zero. Class interval 2 msec. Abbreviations indicate muscle in each case (EDL, extensor digitorum longus; G, gastrocnemius; P, plantaris; S, soleus; TA, tibialis anterior).

DISCUSSION

The fundamental result of the present study is that co-activation of extrafusal muscle contraction and spindle afferent discharge is readily observed on supraspinal stimulation. That is, when stimulation at one of several sites in the brain elicits a contraction in an ankle muscle, many spindles in that muscle are activated and discharge when the contraction begins. This disclosure led to a re-examination of the early discharge of spindles to stimulation of ventral root or peripheral nerve (Granit *et al.* 1959). It proved possible to separate an ephaptic component (cf. Lloyd, 1942; Leksell, 1945), due to excitation of sensory fibres by the muscle action potential which precedes the measurable onset of contraction, from a genuine physiological activation making spindles discharge at the foot of the contraction, just as in the present observations. Our general conclusion is therefore that whatever the site stimulated, be it a place in the brain stem as in this work, or ventral root or nerve as in the second report, evidence for a fast process of spindle activation is obtained; and so it seems necessary to conclude that, essentially, it is one and the same process accessible from different sites. It is not found in all spindles of the leg muscles studied (see Granit *et al.* 1959) and often requires some γ bias or slight extension of the muscle, both procedures known to increase a spindle's phasic sensitivity (Granit & Henatsch, 1956; Lippold, Redfearn & Vučo, 1958). In the periphery, distal to the ventral root, fast spindle activation is conducted at α velocity (Granit *et al.* 1959).

Besides that of Granit & Holmgren (1955), several studies of spindle activation by supraspinal stimulation have been made in the past (Granit & Kaada, 1952; Eldred *et al.* 1953; Eldred & Fujimori, 1958) in which activation has been attributed to excitation of γ fibres. Latency was not measured in the latter studies and the response was described as slowly recruiting in character and was followed by a long-lasting after-effect, although Granit & Kaada (1952) pointed out that activation of spindles from the pyramid caused a quick burst. However, in the experiments of Granit & Holmgren (1955) particular attention was paid to the question of latencies. By stimulating the mesencephalic tegmentum with single shocks they activated the γ loop selectively without producing any contraction. Their average values were of the order of 22 msec (range 20–26) with conduction time to ventral roots L7 and S1 of the order of 7–12 msec. We have confirmed their results in similar experiments. However, we have now demonstrated spindle activation at latencies as short as 14 msec by stimulating the mid-brain at the same site. The difference between the shortest latencies of the two studies can best be explained by the existence of a peripheral mechanism activating spindles at α conduction rates.

In selecting sites for supraspinal stimulation attention was given to regions which send descending fibres to the spinal cord. It was shown that stimulation of the pyramidal tract, which is known to be involved in the production of phasic movements, effectively activated this fast spindle mechanism. Stimulation of Deiters's nucleus and reticular formation, which on the other hand are known to take part in the regulation of postural tone, also evoked an early spindle discharge. The cerebellum has access to these latter regions through fastigio-deitersian and fastigio-reticular connexions, and descending cortical projections to the brain-stem reticular formation have been demonstrated (Rossi & Brodal, 1956). The fast spindle mechanism, then, should be available to several higher centres whose functions are related to regulation of motor activity.

Our results have the further interest that they provide fresh evidence for the existence of a peripheral mechanism facilitating centrally induced muscle contraction. This was demonstrated (Granit, 1950) by using monosynaptic testing to study the excitability of ventral horn cells in response to both shocks and slowly rising currents applied to the peripheral stump of a cut ventral root. Contraction was accompanied by an early rise of motoneurone excitability followed by suppression of excitability as muscular tension increased. The present work and its sequel (Granit *et al.* 1959) suggest that the mechanism behind this brief, early facilitation accompanying contraction is a specific process of spindle activation based on α fibres.

It is not yet known whether the α control of spindles also extends to spinal segmental reflexes. One striking fact seen in work on these reflexes, as pointed out by Hunt (1951), Granit *et al.* (1952), Eldred & Hagbarth (1954) and Hunt & Paintal (1958), is the overwhelmingly strong and well maintained γ effect on extensor spindles from skin receptors. They are clearly organized with a large margin of safety for γ activation and involve enough synaptic delay to make the fast α mechanism less important than the slower γ mechanism. For this reason, even if there be α activation of the spindles at the foot of such reflex contractions, they would be submerged in the powerful discharge set up by the γ system.

SUMMARY

1. The effect of supraspinal stimulation upon the discharge of hind-limb spindle afferents isolated from dorsal roots was studied in decerebrate or anaesthetized cats; tension myograms were simultaneously obtained.

2. Consistent afferent driving was obtained from pyramid, Deiters's nucleus, brain-stem reticular formation and medial longitudinal bundle, using 2–3·5 msec square pulses at one per second.

3. The latency of the spindle afferent response to pyramidal stimulation was generally less than the sum of the average latencies of ventral root response to pyramidal stimulation plus peripheral γ loop from ventral to dorsal root.

4. In almost all cases muscular contraction accompanied afferent driving and the initiation of the spindle response in each case was closely related in time to the onset of the evoked contraction.

5. In view of the temporal relationship between extrafusal contraction and afferent spindle discharge, and the brief latencies observed, it is considered

unlikely that spindles have here been excited by γ motoneurones. Instead, it is suggested that these effects have been mediated by α motoneurones.

6. The physiological significance of fast supraspinal and spinal control of spindles is discussed.

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THE EARLY DISCHARGE OF MAMMALIAN MUSCLE SPINDLES AT ONSET OF CONTRACTION

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There are several papers in which an early mass discharge in the undivided sensory root has been observed upon stimulation of the peripheral stump of severed motor roots (Dun & Feng, 1940; Masland & Wigton, 1940; Lloyd, 1941, 1942; Leksell, 1945; Granit & Skoglund, 1945). Beyond mentioning that such early discharges may come from artificial synapses at cut nerve ends, from ephaptic activation of sensory terminals by the muscle action potential and from muscle receptors, these papers will not now be discussed. Analysis at the single-fibre level, as we shall see, resolves many of the complexities encountered with mass discharges, but it is necessary to be aware of the sources of error elucidated in the papers mentioned.

In the previous paper (Granit, Pompeiano & Waltman, 1959) we showed that co-activation of extrafusal muscle and muscle spindles occurs in response to stimulation of, for example, the medullary pyramids or Deiters's nucleus, so that spikes from large spindle afferents can be picked up in dorsal root filaments early enough to make them coincide in time with the incipient contraction of their muscles of origin. Hunt & Kuffler (1951) had seen an early discharge from spindles when stimulating muscle nerve or ventral root, in fact too early to be caused by γ fibres, and hence raised the issue whether there might not also be more rapidly conducting fibres for the spindles. This they found improbable. Our results with brain-stem stimulation provided evidence for a fast physiological mechanism of spindle activation and thus suggested that the early discharge deserved to be reinvestigated.

This conclusion is underlined by the considerable amount of histological evidence in favour of thicker fibres than those of the γ system running to muscle spindles (see quotations in Granit, Pompeiano & Waltman, 1959). Barker (1948, p. 157), working on leg muscles of cats and rabbits, even concluded that 'in all probability the two polar halves of an intrafusal musclefibre function as independent contractile units', the reason being that he often found one, sometimes two large fibres occupying one pole by themselves. These were 6–7 μ, the smaller ones 3–4 μ in gold chloride stain. Recently Cooper & Daniel (1956) have seen large fibres in the poles of spindles in human hand muscles and have reopened the question as to what extent spindles may receive α motor fibres.

Hunt & Kuffler (1951) found that the early discharge depended upon muscle tension and that its latency (to be called in some contexts 'loop time') could be prolonged up to 6 msec when muscle tension was reduced. It could also be elicited by excitation of motor units, each of which could not be expected to cause potential changes sufficient for excitation by the muscle action potential. Their experiments on root division gave equivocal results (see full quotation below, p. 412). Their final conclusion (p. 310) was that the 'early discharge is caused by tension changes which arise within the muscle before they are recorded myographically'. As a serious argument against concluding that the early discharge is an α activation they also regarded the fact that it was seen in both the *A* (spindles) and *B* (Golgi tendon) organs of Matthews (1933). We feel, on the contrary, that this would be a consequence of genuine intrafusal fast activation. The spindles, though in parallel with muscle, have their afferent endings in series with intrafusal muscle, just as the tendon organs are in series with extrafusal muscle. Therefore both should be activated by contractions in their respective muscular elements. However, the extrafusal contraction may well delay the moment at which an intrafusal contraction can activate its sense organ, as was emphasized by Granit, Pompeiano & Waltman (1959) and shown to hold good for γ activation (their Fig. 1). In their case the onset of γ activation, as measured by a spindle discharge, was accelerated by selective removal of the extrafusal contraction with Flaxedil (gallamine triethiodide; May and Baker, Ltd). In preferring a tension theory to what might be called an 'innervation theory' Hunt & Kuffler's chief support came from experiments on root division (to be discussed below).

Spindles are length receptors inserted so as to make their two poles approach each other in extrafusal contraction. They will thereby unload the equatorial region containing the sense organ on the nuclear bag. This should now be well known. Therefore the evidence for quite the opposite mechanism, implied in the 'tension theory' of Hunt & Kuffler (1951), has to be particularly compelling to be conclusive, even when it is granted that some spindles may be displaced and pulled upon by contraction in adjacent extrafusal bundles. In order to exclude freakish effects it is necessary to have some idea of the distribution of latencies and the regularity with which the early discharge occurs at different extensions in muscles of different twitch times. In the present work we have studied 168 spindles in both slowly and rapidly contracting muscles, extensors as well as flexors of cats, and made a number of supplementary observations on rabbit spindles which behave similarly. In addition we have studied 19 Golgi tendon organs.

It should at the outset be realized that increased extension of a muscle may cause two antagonistic effects. (1) It will improve the phasic sensitivity of the spindles (Granit & Henatsch, 1956; Lippold, Redfearn & Vučo, 1958), which is important in fast activation. (2) It will cause larger contractions likely to unload spindles more effectively than contractions from zero extension. This would be expected to delay activation. The balance point of these two antagonistic effects may be very different in different spindles.

METHODS

This is a continuation of the previous work on fast supraspinal activation of spindles (Granit, Pompeiano & Waltman, 1959), in part even making use of the same animals. A few were decerebrated, the others were anaesthetized with 20 mg chloralose and 10 mg pentobarbitone per kilogram. In both types of experiments a stimulating electrode was placed on the appropriate muscle nerve in the popliteal space, at knee level, both in order to determine conduction velocity in the spindle afferent isolated in a dorsal root filament and to study the response to contraction. The term 'slack muscle' means that the muscle is unhooked from the myograph. Another pair of stimulating electrodes, when used at all, was placed on the peripheral stump of the cut ventral root L7 or S1. In many experiments root stimulation was not employed; the ventral roots were then intact.

RESULTS

Comparisons of different muscles. Loop time. The first question concerns the percentage of spindles capable of discharging early spikes when the shock is delivered to the nerve at knee level. This was found to be for soleus 77 % out of a total of 31, for gastrocnemius 50 % of 52, for tibialis anterior + extensor digitorum longus 40 % of 85. Now, time to peak of twitch is around 60 msec for soleus, around 30 msec for gastrocnemius and below 20 msec for tibialis anterior (Cooper & Eccles, 1930; Gordon & Phillips, 1953). Extensor digitorum longus is also a fast muscle with contraction times of the order of those found in tibialis anterior. Thus, what our results show is that in the slow soleus the early discharge is far more regularly obtained than in the fast flexors. This, of course, is quite the opposite of what a tension theory would require. Early in contraction there is far more tension produced in fast muscles.

Figure 1A serves as a first introduction to what proved to be the major obstacle to an understanding of this problem. Records 1 and 2 show the early spike in a slack soleus at two shock strengths. The first spike is the direct spike (DS), slightly interfered with by some other spikes from the gastrocnemius nerves, the electrode being at the knee. In 1 the latency of the earliest spike from muscle (ES), is long. The stronger shock in 2 brings in a still earlier spike that is now our ES, but the other one is found in its former temporal position. In 3 and 4 the same experiment is repeated with the muscle hooked to the myograph. Our original ES in 1 is now doubled and has a slightly shorter latent period. With the stronger shock in 4 we again obtain the ES of record 2. There were thus, in this experiment, two types of ES, one isolated in record 1 being of low threshold and being doubled in 3 and 4 when the muscle is extended, while the other, seen only with the slightly stronger shocks in 2 and 4, has a very brief latent period, and in this particular case requires the number of α fibres excited by the stronger shock, but neither any extension nor the tension produced by the shock.

Figure 1B has been put in merely to confirm the finding by Hunt & Kuffler (1951) that the very early spike can also occur in a Golgi tendon organ. After a pause this organ responds to tension in accordance with the definition of its properties (Matthews, 1933). Figure 1 also shows that the first type of ES occurs before visible contraction in a myogram, the second at the foot of the contraction. Both types are seen in extensors, but practically the latter type alone in flexors. For this reason it was of considerable interest to study the distribution of ES latencies in our material.

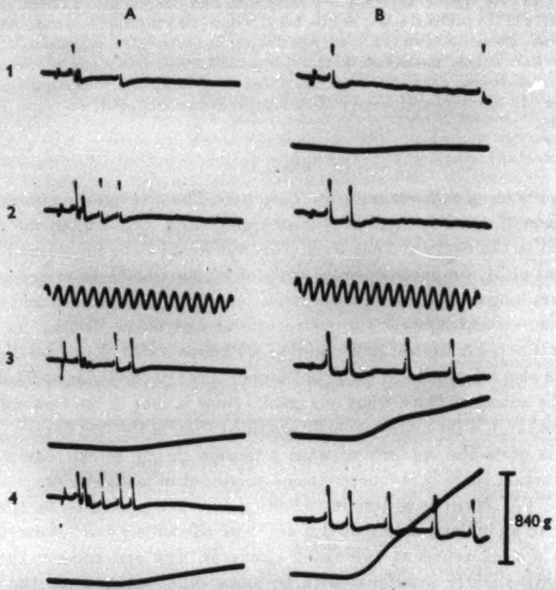

Fig. 1. A: Soleus spindle afferent in anaesthetized cat. Nerve stimulation. 1, shock strength 1·3 times threshold for direct spike, slack muscle; 2, same at 1·6 times threshold. Earliest spike in 1 at latency 5·2, in 2 at 3·4 msec; second spike in 2 at 5·2 msec. 3 and 4 at 15 mm extension and shock strengths 1·3 and 2·0 times threshold respectively. Conduction time of direct spike is 1·4 msec. B: Gastrocnemius tendon organ afferent in anaesthetized cat. Nerve stimulation. 1, at threshold for direct spike, conduction time 1·3 msec; 2, at 1·6 times threshold, slack muscle; 3, same strength at 4 mm extension; 4, same at 10 mm extension. Earliest spike at 3·2 msec. Time 1000 c/s. Calibration of myograph in this and all successive records marked for 1 cm on original film.

Within the early discharge we have measured the time to the ES. From this value has been subtracted the conduction time from knee level to dorsal root of the direct spike (DS). In the histogram of Fig. 2 loop time is ES–DS which, accordingly, refers to the loop traversed from nerve electrode at the upper edge of the gastrocnemius muscle, round the muscle and back to nerve electrode. Only five flexor spindle responses were of the type conducted in slow afferents (40–60 m/sec). Three of them had no ES, the other two had loop times of 5·2 and 6·5 msec respectively. The rest, 163 spindles, were of the nuclear bag variety. Figure 2, of course, only includes the spindles that had an ES. No differences were noted in the conduction velocities between spindles and tendon organ afferents, which agrees with the results of Hunt & Kuffler (1951).

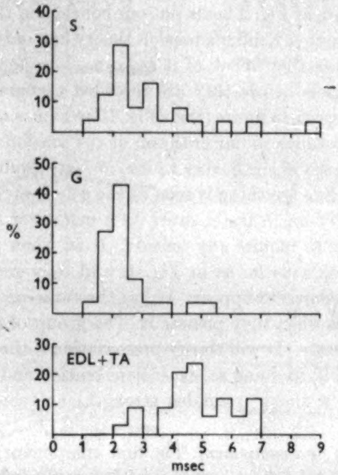

Fig. 2. Percentage distribution of loop time. From above downwards: 24 spindles in soleus, 26 spindles in gastrocnemius, 34 spindles in tibialis anterior and extensor digitorum longus.

The histogram shows that loop times in gastrocnemius were short and well concentrated within a narrow range. Some in soleus occupy the same range but others spread out into longer values. In the flexors, only five ES had short loop time, the others had long ones, but no longer than the longest in soleus. It is impossible to ascribe all these differences to variation in conduction times.

Gastrocnemius and tibialis anterior have very similar efferent calibre spectra for large fibres (Rexed & Therman, 1948). Extensor digitorum longus is likewise very similar to gastrocnemius (Eccles & Sherrington, 1930). Only soleus has efferent α fibres considerably thinner than the others (Eccles & Sherrington, 1930). Even if the long loop times in soleus were attributed to this factor, there remain to be explained the equally long loop times in the flexors. In our experiments with electrodes at the upper edge of gastrocnemius there is a further 3 cm to this muscle and soleus, and 6–8 cm to the flexors to account for. This, at 100 m/sec, would add around 1·0 msec to the flexor loop time ($8-3 = 5$ cm, twice over) which is insufficient to explain the fundamental differences between flexors and extensors. Below it will be shown that the distribution of loop times hinges upon other factors.

Figure 1 has already given clear indication of the basic disclosure of our analysis, namely that the early discharge is a heterogeneous event within which two components clearly stand out. One is the spike of very short loop time, visible before contraction, the other is the spike at the foot of the contraction. The diagram of Fig. 2 bears out our conclusion that the former spike is rare in flexors. Hunt & Kuffler's tension theory can only refer to the former type of spike, because they speak of it as 'caused by tension changes which arise within the muscle before they are recorded myographically'. However they picture both types in a figure (their Fig. 10) which is not unlike our Fig. 1.

Figure 2 adds substance to our criticism of the tension theory. Why is the very early spike so rare in the flexors which, in fact, produce visible tension a good millisecond before anything is seen in the soleus myogram and, furthermore, do so at a very much faster rate? As a matter of fact, we do not find this very early spike to require any tension. It is, when present at all, regularly present in slack muscle, as in Fig. 1, and only requires a sufficiently strong synchronous shock to appear. It has the same property in the flexors in the rare instances when they possess it. The group of slightly later spikes behaves very differently. It will clarify presentation if the early component is dealt with first, and if, in doing so, extension, tension and number of efferent fibres activated by a shock (stimulus strength) are considered separately, whenever possible.

Ephaptic nature of first component. The first component, if elicited at near-threshold strength, is sensitive to extension. It may disappear when the muscle is pulled out. When the shock is strong the spike generally resists this effect of extension. However, sometimes no amount of stimulus strength can prevent the disappearance of the first component of ES.

In Fig. 3 are presented two such extreme cases of the effect of extension. It should be noted that there is only one ES and no later burst. A is a gastrocnemius spindle stimulated at knee level. In 1, at zero extension, the DS is shown. Some increase of stimulus strength in 2 adds the ES, shown in 3 at 5 mm extension. In 4, at 8 mm extension, it is gone but returns in 5 when the muscle is made slack. This experiment was next repeated with maximal shocks and the electrodes lifted by hand so as to pull slightly on the nerve and make good contact. The same result was obtained. In B1 is the DS from another gastrocnemius spindle. B2 is excitation from the ventral root for calculation of conduction velocity (see legend and below). In B3 a strong stimulus is used, as is also seen by the fact that other spikes add themselves to the DS functionally isolated. Records B3 and B4 are at zero and 5 mm extension respectively. With 11 mm extension, in B5, the ES is gone. Thus, contrary to the tension theory, an ES may disappear when the muscle is extended and produces more tension. Again it should be noted that the early spikes studied in Fig. 3 were extensor spikes of very brief loop time not followed by a burst at any stimulus strength or extension. It is pertinent to ask: why no later burst despite a great deal of tension? We shall provide good evidence for our preliminary conclusion that this is because the second component of ES is absent in these particular spindles.

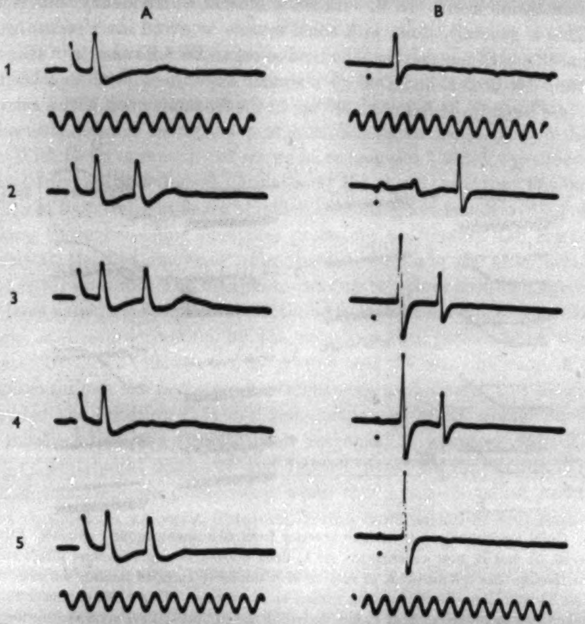

Fig. 3. Two spindle afferents from gastrocnemius of anaesthetized animals. Stimulating nerve. A. 1, direct spike, conduction time 1·2 msec; 2, strong shock elicits early spike at latency 3·5 msec. Zero tension; 3, same at 5 mm extension; 4, at 8 mm extension early spike disappears; 5, return of early spike in slack muscle. Loop time, which is ES–DS, equals 2·3 msec. B, another similar experiment. 1, direct spike, conduction time 1·2 msec: 2, stimulation from ventral root, spike after 5·0 msec; 3 and 4, strong shock to nerve elicits early spike from slack muscle with perfect regularity and augments direct spike by component of unknown origin. Latency 3·7 msec. 5, disappearance of early spike when muscle extended 11 mm. Loop time 2·5 msec. Nerve latency subtracted from root latency gives 1·3 msec or 115 m/sec, conduction distance being 15 cm. Time 1000 c/s.

Since the first component (see Fig. 1) also occurs in Golgi tendon organs, its properties are next (Fig. 4) analysed in two such preparations. A 1 shows the ES in slack muscle. A burst follows when the muscle picks up tension (A 2). In A 4 the ES is absent at 10 mm extension and is but irregularly present at 9 mm extension in A 3. In B, with slack muscle, a 'frequency test' is carried out. This is generally done with slack muscle to avoid interference with the genuine effects of contraction. The tendon organ ES follows rate of stimulation to around 160/sec. C and D show a similar experiment with another tendon organ (see legend). It followed 200/sec in the frequency test with slack muscle (C 4). The ES, so far from being favoured by tension, disappeared when tension increased as a consequence of applied extension (D 2 and 3).

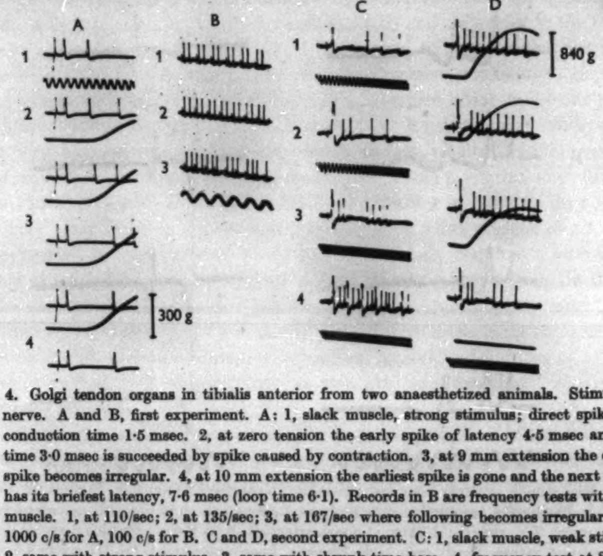

Fig. 4. Golgi tendon organs in tibialis anterior from two anaesthetized animals. Stimulating nerve. A and B, first experiment. A: 1, slack muscle, strong stimulus; direct spike with conduction time 1·5 msec. 2, at zero tension the early spike of latency 4·5 msec and loop time 3·0 msec is succeeded by spike caused by contraction. 3, at 9 mm extension the earliest spike becomes irregular. 4, at 10 mm extension the earliest spike is gone and the next in turn has its briefest latency, 7·6 msec (loop time 6·1). Records in B are frequency tests with slack muscle. 1, at 110/sec; 2, at 135/sec; 3, at 167/sec where following becomes irregular. Time 1000 c/s for A, 100 c/s for B. C and D, second experiment. C: 1, slack muscle, weak stimulus. 2, same with strong stimulus. 3, same with shrunk time base. 4, frequency test at 200/sec, where following still occurs, loop time (4·0–1·7) being 2·3 msec. Records in D, 1–3, to show disappearance of earliest response at higher extensions. 1, 4 mm; 2, 15 mm (spike gone); 3, 17 mm; 4, control with slack muscle. Early spike again present. Time 1000 c/s.

The frequency test with slack muscle always succeeds and does so equally well with the similar ES component in muscle spindle afferents (see below, Fig. 6). Tendon organs in a flexor have been chosen for the particular reason that the frequency test fails with ES in flexor spindles except in the rare cases when the ES occurs before visible contraction (see below). All Golgi tendon organs, by definition (Matthews, 1933), will have the secondary burst in response to tension and so from them one may obtain the loop time for a possible genuine effect on sense organs by contracting muscular tissue. It is, for instance, 6·1 msec in Fig. 4A.

It is evident that genuine activation by contraction is dependent upon two factors, threshold of end organ and contraction itself. If spindles have a genuine mechanism, based on fast contraction, of the order of velocity seen in the surrounding extrafusal fibres from which they ontogenetically originate (Cuajunco, 1927), loop times may be slightly shorter because spindles tend to have lower thresholds than tendon organs (Matthews, 1933; Hunt & Kuffler, 1951). With Golgi organs in flexors we have had loop times for the secondary discharge from 4·2 to 8·5 msec. A glance at Fig. 2 will show that this includes the range of loop times in flexor spindles, excluding five spindles (6 %) of very brief loop times belonging to spikes preceding contraction and responding positively to the frequency test. For this test values of the order of 130/sec tend to be a minimum. The maximum runs up to values around 250/sec.

We have called these spikes ephaptic because their most likely mechanism of origin is direct activation by the muscle action potential, as was well elucidated by Lloyd (1942) when he showed that the mass discharge of dorsal root spikes follows the fast stimulus frequencies used above. To this we have only added the criterion that our first component of ES should do this in slack muscle. Leksell's (1945) 'back response' is identical with Lloyd's secondary centripetal discharge and Leksell gives one illustration (Fig. 28, p. 56) of this response diminishing when the muscle is pulled upon. The efferent conduction velocity, obtained from a comparison of root with nerve stimulation, is always in the upper α range (see legend of Fig. 3).

Comparison with muscle action potential. Our conditions have not been favourable for recording the muscle action potential, but Eccles & O'Connor (1939) have devoted a careful study to this very problem in two of the muscles we have used, soleus and tibialis anterior. Their electrode distance was 1 cm, ours averaged 3 cm for the extensors and 7 cm for the flexors (see above). In soleus the muscle action potential occurred after a delay that was usually 0·6, occasionally 1·0 msec. In our case, at 3 cm, the longer value is likely to be the better choice. The negative potential wave at the end-plate region lasted 2 msec in soleus, 1·5 msec in tibialis anterior. The longer efferent conduction distance in the flexors we assume to be roughly compensated for by the faster conduction velocity in comparison with soleus. The muscle action potential will stimulate on the rising phase, in favourable cases a second time on the falling phase (double spikes are seen from time to time). Since loop times are calculated with reference to the nerve electrodes it is necessary to add afferent conduction times of approximately 0·2 msec for soleus and 0·5 msec for the flexors. Thus loop times below 2·5 msec must be ephaptic and very likely, with our electrode arrangements, 3·0 msec is a critical duration in extensors, 3·5 msec in flexors. For practical purposes the frequency test, when positive, means that such spikes cannot be considered relevant to our problem, which concerns the possibility of genuine activation. This problem presents itself in pure form in most flexor spikes for which, also, the briefest loop time of Golgi tendon organs, 4·2 msec, is in good agreement with our measurements for spindles (Fig. 2), if 0·5–1·0 msec is added for initiation of the contraction, be it then intra- or extrafusal. The sense organs themselves have extremely high phasic sensitivity when extended, or under γ bias (Granit & Henatsch, 1956) and will respond to elongations at the rate of a few microns per millisecond.

Early spikes activated by contraction. The genuine early discharge is seen in both extensors and flexors, though in the former more often than not complicated by the ephaptic spike. Figure 5 shows the separation of the two components for a soleus spindle afferent, stimulated from the root in order to exclude reflex interference and pull upon the muscle nerve (for anatomical reasons less of a complication with flexors). In this case the records at different extensions are at threshold, (1), at 1·7 times threshold to show the range of variation in 2 and 3, and at 3·3 times threshold in 4. The earliest spike cluster, whose pattern is rather variable, probably comes from an unidentified muscle near the spine; less probably, from an artificial synapse in the cut hamstring nerve (cf. p. 399). However, the regular spikes are clearly defined. A shift of loop time for the ES is seen when the muscle is lengthened, and though occasionally at 5–10 mm loop time reverts to short values, it never does at 15 mm. This means that for this particular figure we have chosen a spindle of the type analysed above, characterized by an ephaptic spike sensitive to extension. The ephaptic spike has disappeared at 15 mm extension. As stated,

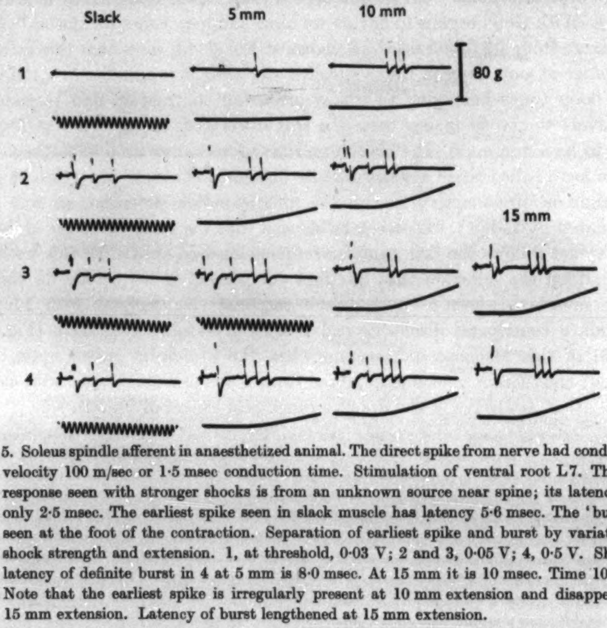

Fig. 5. Soleus spindle afferent in anaesthetized animal. The direct spike from nerve had conduction velocity 100 m/sec or 1·5 msec conduction time. Stimulation of ventral root L7. The first response seen with stronger shocks is from an unknown source near spine; its latency was only 2·5 msec. The earliest spike seen in slack muscle has latency 5·6 msec. The 'burst' is seen at the foot of the contraction. Separation of earliest spike and burst by variation of shock strength and extension. 1, at threshold, 0·03 V; 2 and 3, 0·05 V; 4, 0·5 V. Shortest latency of definite burst in 4 at 5 mm is 8·0 msec. At 15 mm it is 10 msec. Time 1000 c/s. Note that the earliest spike is irregularly present at 10 mm extension and disappears at 15 mm extension. Latency of burst lengthened at 15 mm extension.

spindles have this property if studied at shock strength near threshold, in confirmation of which the genuine burst is seen alone also at 10 mm in the uppermost line of Fig. 5. Its latency varies a little with stimulus strength. Extension also is important. Latency is shortest at 5 and 10 mm (8·0 msec), line 4. Tension rises faster and to much greater values at 15 mm where the latency is 10 msec, lines 3 and 4, in agreement with our conclusion that the rising tension, so far from favouring the appearance of the spike, actually delays it. This need not, however, always be the case because, as already pointed out (p. 400), the equilibrium point between the favourable effect of extension on phasic sensitivity and the unloading of the spindle by the increase in extrafusal tension is unpredictable. In the present case the favourable effect of extension is on the number and frequency of the spikes in the burst. Tension delays the process. This is commonly seen.

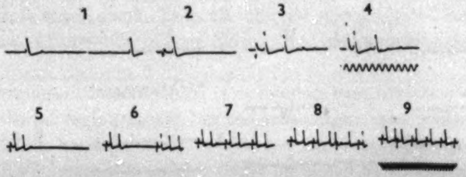

Fig. 6. Stimulation of nerve to study response of same spindle as in Fig. 5 to shock frequencies. Slack muscle. 1, spontaneous activity; 2, direct spike of conduction time 1·5 msec at threshold strength, 0·63 V; 3, at 1·0 V; 4, at 6·3 V with time 1000 c/s. Latency of earliest spike in slack muscle was between 4·2 and 4·5 msec, hence loop time 2·7 msec; 5–9, time base shrunk for frequency test at 1·0 V. 5, very slow frequency; 6, 60/sec; 7, 110/sec; 8, 155/sec; 9, 200/sec and time 1000 c/s. Note lengthening of latency and failure of driving beginning in 8. (Fig. 7, A4, shows failure of frequency test with flexor spindle.)

Figure 6 completes the argument by showing the result of the frequency test with the ephaptic spike of this particular spindle. The figure is fully explained in its legend.

While Figs. 5 and 6 serve to show how it is possible in extensors to separate the ephaptic spike from the genuine effect, our own procedure in studying the latter has been to take advantage of the natural isolation of the genuine process in flexors. In them it is not very common to find the early second component of the discharge in slack muscle. Some flexor spindles show it at zero initial length, others require some extension. Having noticed that the 'burst' of early spikes tended to be more prominent in the active spindles of extensors in the decerebrate preparation, we tried to raise the γ bias of flexor spindles which failed to give early spikes in slack muscle. Such experiments are shown in Fig. 7. A1 illustrates the DS in slack muscle. There was no ES. Then some manipulation of the limb started activity from the spindle and if the shock was fitted into this discharge, the spindle responded with an ES (A2).

Record A3 was put in to demonstrate that this ES belonged to the characteristic flexor type, discharging at the foot of the contraction; record 4 shows that it did not follow frequency 67/sec. The records B begin with slack

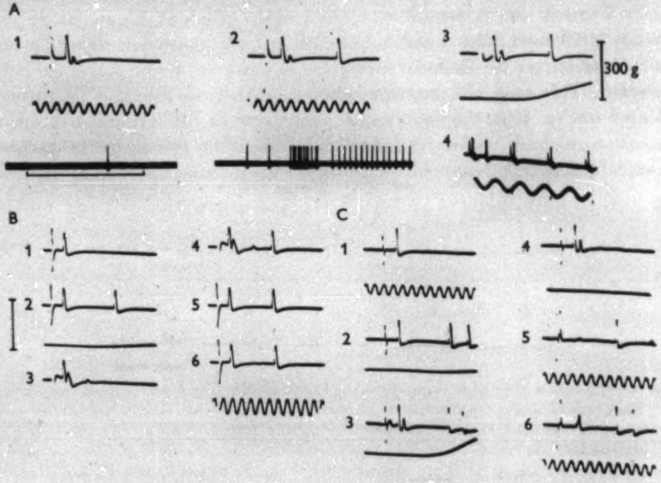

Fig. 7. Spindle afferents from flexors. Anaesthetized cats. Stimulating nerve. A, extensor digitorum longus. *Above*, records on sweep circuit and time 1000 c/s; *below*, simultaneous records on standing spot, 0·5 sec marked below middle record. 1, only direct spike of conduction time 1·5 msec when spontaneous activity low in slack muscle; 2, an early spike of latency 6·5 msec could be elicited at same shock strength after starting spindle activity by slight manipulation of leg; 3, contraction at zero extension to show position of spike relative to myogram—spike always present at zero; 4, only direct spike followed frequency 67/sec at this extension, nor did early spike in slack muscle, even when activated, follow frequencies between 25 and 50/sec. B, extensor digitorum longus; calibration of myograph as in A. 1, slack muscle, direct spike of conduction time 1·3 msec. Shock strength 0·32 V; 2, same at zero extension elicits early spike of latency 7·0 msec; 3, back to slack muscle but now shock strength 1·0 V, in spite of which no early spike; 4, after a 1·5 sec tetanus at 67/sec to stimulate γ fibres there is now an early spike in slack muscle at 1·0 V; 5–6, cutting down stimulus strength to original 0·32 V; this spike was seen in slack muscle for some time after the tetanus. Latency now 6·0 msec. Time 1000 c/s. C, tibialis anterior. 1, slack muscle, direct spike of conduction time 1·4 msec; 2, zero extension, shock strength 0·1 V, early spike at latency 7·0 msec; 3, shock strength now 0·25 V; 4, same in slack muscle, no early spike, nor could one be elicited by increasing shock strength; 5, very weak stimulus below strength for DS through another stimulator, then tetanus of popliteal nerve for flexor reflex on separate stimulator; records taken after tetanization during reflex in γ motoneurones; 6, repetition of 5 to demonstrate occasional double responses. Early spike in 5 and 6 has latent period of 6·0 msec and loop time of 4·6 msec, thus shorter than at zero extension in spite of muscle being kept slack from 4 to 6. Neither by flexor reflex nor by any amount of extension could latency be pushed below 6·0 msec.

muscle in which (in 1) only a DS is seen, but at zero extension in B 2 an ES can be obtained at time 7·0 msec from shock artifact. Yet in B 3, when the muscle was again unhooked from the myograph, a 3·3 times stronger shock (eliciting another component spike in the filament) failed to raise an ES. The nerve was therefore briefly tetanized at this strength (suprathreshold for γ fibres) and immediately afterwards, in 4, an ES was obtained which in 5 and 6 was shown to require only the weaker shock of record 1, yet had a shorter latency, 6·0 msec. Activation could not in this case be obtained by tetani just maximal for extrafusal contraction. Finally, records C illustrate a case of spindle activation by a (popliteal) flexor reflex. Record 1 is the DS; in 2, at zero extension, a weak shock elicits a burst; in 3 a stronger shock shortens the interval between the pair of spikes in the burst but fails to have any effect in 4 when muscle is made slack. Then the spindle was activated reflexly to increase its γ bias. Afterwards, in 5, an ES occurred in the slack state, sometimes double (6).

From experiments of this type it is concluded that intrafusal pull on the spindle's equatorial region containing the sense organ may replace extension of the muscle and, hence, that extrafusal tension cannot be regarded as the decisive factor whenever it is automatically increased by an increase of stimulus strength or extension. Sufficient activation of the spindle itself may often make it discharge an early spike or even a burst of spikes in slack muscle. These results were confirmed in a number of experiments in which the early spike was lost after de-efferentation (preceding root stimulation) but could be re-activated by post-tetanic potentiation at sufficient strength. It is clear that, since spindles differ widely in their sensitivity to γ stimulation, activation by various means does not always succeed. Occasionally a tetanus may also silence a spindle (Kuffler, Hunt, & Quilliam, 1951; Granit, Homma & Matthews, 1959).

Flaxedil has been used in this laboratory with mammals (Granit, Homma & Matthews, 1959; Granit, Pompeiano & Waltman, 1959) to suppress extrafusal contraction with preservation of good γ reflexes, and the same result has been obtained independently by Henatsch & Schulte (1958) with frog muscle spindles. Since in frogs α activation of spindles also is spared by Flaxedil (Henatsch & Schulte) it was used here at the end of several experiments with the early discharge. This generally disappeared before extrafusal contraction was fully gone, but could be restored by post-tetanic potentiation and then, on account of the smaller and more slowly rising extrafusal contraction, sometimes with shorter latency than in the controls before Flaxedil. The ephaptic spike was more sensitive to Flaxedil and less so to post-tetanic potentiation than the physiological response at the foot of the contraction (cf. Lloyd's (1942) results with curare on his ephaptic mass response). However, the arguments against the tension theory and for the differentiation of the early discharge into two components, neither of which depends on tension, are cogent enough without burdening this paper with additional illustrations from the Flaxedil experiments.

Root division. The experiments of Hunt & Kuffler (1951) on root division sometimes showed that none of the individual parts gave an ES when several put together produced one, suggesting to us that they studied an ephaptic spike requiring a great many synchronously activated fibres. 'In some experiments, on the other hand, an early discharge was obtained on stimulation of a whole ventral root and of several of its subdivisions' (p. 310). It is difficult to interpret root records without clearly separating the two kinds of mechanism that are responsible for early spikes and without considering what γ activation might do to subdivisions provided with a large number of γ fibres as compared with filaments lacking them. To use flexors and apply post-tetanic potentiation is to play for safety.

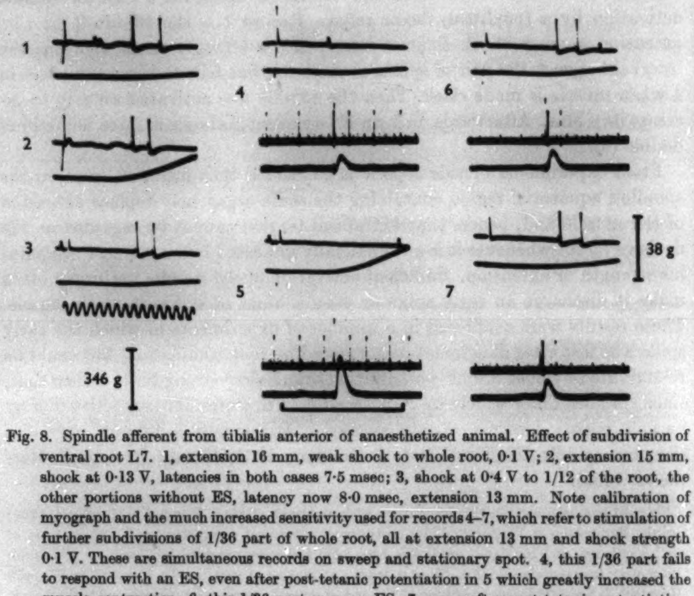

Fig. 8. Spindle afferent from tibialis anterior of anaesthetized animal. Effect of subdivision of ventral root L7. 1, extension 16 mm, weak shock to whole root, 0·1 V; 2, extension 15 mm, shock at 0·13 V, latencies in both cases 7·5 msec; 3, shock at 0·4 V to 1/12 of the root, the other portions without ES, latency now 8·0 msec, extension 13 mm. Note calibration of myograph and the much increased sensitivity used for records 4–7, which refer to stimulation of further subdivisions of 1/36 part of whole root, all at extension 13 mm and shock strength 0·1 V. These are simultaneous records on sweep and stationary spot. 4, this 1/36 part fails to respond with an ES, even after post-tetanic potentiation in 5 which greatly increased the muscle contraction; 6, this 1/36 part gave an ES; 7, same after post-tetanic potentiation. Note that in spite of considerably greater tension developed in 5, the ES only occurred in 6 and 7, latency 8·0 msec. Time 1000 c/s for sweep and 0·5 sec marked for standing spot.

With some patience one may succeed in finding a small fraction of a root such as the 1/12th used in Fig. 8, record 3. This was the only one that had the ES, which was also seen after the whole root was stimulated (1 and 2). This 1/12 part was divided into three roughly equal parts, each of which therefore represented about 1/36 part of the original root (L7). Two of these 1/36 parts failed to produce an ES, despite potentiation at maximal strength, after which they were tested at all strengths short of values producing stimulus spread. In the figure the one 1/36 part has been picked out that gave a contraction (records 4 and 5). The third 1/36 part produced an ES (6 and 7) whose latent period, after post-tetanic potentiation (7) was reduced to 8·0 msec. The important point of this experiment is that the potentiated contraction not only was very small but considerably smaller than in the 1/36 part that failed to produce an ES. Therefore tension as such cannot explain the result. The ES is due to the action of a specific fibre or set of fibres.

The most general result of root division in flexors was that increase of stimulus strength or adding up thin filaments can reduce the latent period of the ES (after those parts of the root had been set aside which failed to produce an ES). With a suitable fraction of the root this result could be obtained without significant increase of contraction, whereas in other cases contraction did increase. Such experiments only show that number of fibres activated plays a part and hence support the notion of some overlap of the fibres concerned. If increase of tension is singled out as the only possible explanation of the effect of increased shock strength or addition of fibres from other filaments, this conclusion is open to the criticism that the connexion may be fortuitous (cf. Fig. 8), due merely to the fact that increase of number of efferent fibres is often equivalent to an increase of tension. For this reason it is held that the clarity which is supposed to be the reward of experiments on root division often is more apparent than real. It should be noted that the first and major part of Hunt & Kuffler's (1951) experiments on root division deals with spikes much later than the early discharge.

Conduction velocity. Figures 1 and 5 are instances of many experiments which have shown that the physiological early discharge, our second component of ES, can be obtained at shock strengths a good deal below those necessary for maximal extrafusal contraction, which in turn is a good deal below those necessary for the activation of γ fibres (Leksell, 1945; Kuffler *et al.* 1951); not to mention that the large majority of γ fibres require to be repetitively stimulated in order to exert any effect at all on the muscle spindles as studied in the afferent discharge, particularly when the muscle is slack (Hunt & Kuffler, 1951; Kuffler *et al.* 1951).

It would seem a simple matter to supplement these observations, convincing in themselves though they be, with direct measurements of the increase of latency when the shock is shifted from nerve to root. However, the previous sections have shown that the latent period of the ES is influenced by extension, is often delayed by the development of tension and may be shortened by intrafusal activation, etc., factors which our results show to be differently balanced when root and nerve stimulation are compared. The error introduced by measuring conduction velocity across the loop is of the order of 1 msec but, if at the available conduction distance the velocity be 100 m/sec, an error of 1 msec lengthening of latency will reduce the measured conduction velocity to

60 m/sec! One of our best experiments is reproduced in Fig. 9, and is explained in its legend. From the root the average latency of a large number of such records was 8·0 msec, from the nerve 6·8 msec. The difference of 1·2 msec corresponds to a conduction velocity of 125 m/sec. This value seems too large, but no possible error could bring it down to γ conduction velocity.

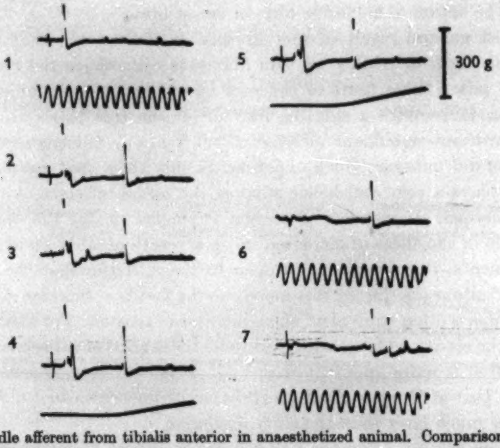

Fig. 9. Spindle afferent from tibialis anterior in anaesthetized animal. Comparison of root and nerve stimulation to estimate conduction velocity. *Nerve.* 1, muscle slack, shock at 0·12 V; 2, same at 0·2 V; 3, same at 1·0 V; 4, 8 mm extension, shock at 0·25 V; 5, same at 11 mm extension. *Root.* 6, shock to L7 at 0·32 V, muscle slack; 7, same at 8 mm extension. Difference in latency, root − nerve, is 7·98 − 6·78 = 1·2 msec, based on measurements from a large number of similar sweeps. Conduction velocity 125 m/sec.

In another set of three experiments the conditions were improved by using two electrodes 5 cm apart on the nerve and taking alternate readings, in one of them alternating rapidly by means of a switch. These values averaged 101 m/sec, with 143 and 73 m/sec as the extremes. All these observations refer to flexors. The ephaptic spike which can be measured in slack muscle was found to be activated by fibres in the upper range of α conduction velocities (cf. legend of Fig. 3).

DISCUSSION

Lloyd's (1942) work and some observations of Leksell (1945) clearly showed that the ephaptic theory cannot be neglected; indeed, ephaptic spikes even turn up in ventral roots. By the frequency test with slack muscle, supplemented with measurements of loop time, we have proceeded to eliminate early spikes which can be explained in this manner, and so they can also be dismissed from this discussion. As far as the present work is concerned, these ephaptic spikes may well contain an unknown fraction of 'physiological' spikes which our criteria fail to differentiate. Also the border line between the two components of early discharge need not always be readily defined. In the flexors the nerve fibres enter fanwise over a considerable distance, while in the two extensors their mode of entrance suggests precise foci, as were proved to exist for the soleus by Eccles & O'Connor (1939), who for this very reason used soleus in their study of the muscle action potential. This may provide one explanation of the differences between flexors and extensors, possibly not the only one.

The remaining theories are (i) the tension theory of Hunt & Kuffler (1951), (ii) the innervation theory and (iii) what one might call an organization theory. The tension theory of Hunt & Kuffler was developed to account for the spikes occurring before visible contraction, which were dismissed for the reasons mentioned above. This does not exclude another tension theory to explain our results with the 'physiological' second component in the early discharge. However, section by section the tension theory has been put to a test and always failed to explain our findings. It may well hold for exceptional spindles but for these no theory is required.

The organization theory is an attempt to rescue the tension theory by the additional assumption that a few extrafusal fibres are specifically connected to spindles in such a manner as to pull upon them when contracting. It thereby avoids the difficulty that, as a rule, tension is of little importance compared with extension and intrafusal state of tonic contraction. The theory postulates a specific anatomical mechanism of organization to explain what otherwise would be a deficiency on a pathological scale (40% in flexors) in a measuring instrument designed to maintain a great measure of independence from extrafusal contraction. All our results, however, go to show (cf. Granit, Pompeiano & Waltman, 1959) that the second component of the early discharge is part of a physiological mechanism. The organization theory would require further specifications to account for the fact that these spindles actually are unloaded, just as those lacking the early discharge, when tension reaches full development. There is no definite evidence in favour of the organization theory. In this regard the innervation theory has the advantage of anatomical support (cf. p. 399).

Our previous experiments on stimulation from central stations such as the medullary pyramids and Deiters's nucleus showed that the mechanism of fast activation gave spikes arising with the muscle contraction. Most of them were too late to be ephaptic. Besides, central stimulation produces a burst spread out further in time by the large conduction distance, and so will not regularly be able to create the electrical fields that synchronous shocks to the nerve set up. The contractions from the pyramids, as a rule, were very small.

Nevertheless, since in no instance could fast activation be obtained from places that did not also potentially excite large extrafusal fibres, it is possible that the fast fibres for the spindles are extrafusal ones which send a tapering branch to a spindle. This alternative is suggested by some anatomical observations of Cooper & Daniel (1956).

Cooper & Daniel (1956) have also suggested other types of nerve ending on intrafusal muscle which by degeneration experiments (Boyd, 1959) have been shown to be motor. There are thus two types of motor endings on intrafusal muscle. Pascoe (1958) has described a spindle efferent, stimulated by violent reflex movements but stretch-insensitive, whose conduction velocity is not given in the paper but which (personal communication) appears to exceed that of γ fibres. At the moment it is impossible to say more than that these observations will eventually have to be evaluated relative to the findings in this paper.

Finally Boyd (1959) has differentiated slow and twitch-like contractions in spindles by direct observations under the microscope on tenuissimus in cats. The effects we have described do behave as if they were elicited by brief intrafusal twitches. The slow contraction suggests the slow mechanism of γ activation. Adding all these observations to the histological demonstrations of spindle motor fibres of two calibres (p. 399) it is clear that our highly specific effects are best explained by an innervation theory. This implies that a considerable number of spindles possess α innervation, often in combination with γ innervation. The functional significance of fast spindle activation has been discussed in our previous paper (Granit, Pompeiano & Waltman, 1959) and, within limits, is independent of whether the α fibres concerned go to spindles alone, to extra- and intrafusal muscle, or only to nearby extrafusal fibres to which spindles are inserted so as to receive a brief twitch while the full force of the extrafusal contraction later is neutralized.

SUMMARY

1. Muscle spindle afferents, coming from ankle flexors or extensors (cat) and isolated in thin dorsal root filaments, discharge early in contraction to a shock to the muscle nerve or the severed ventral root.

2. This early discharge was found to consist of two components, one appearing in advance of visible contraction, the other one at the foot of the contraction. The properties of the two components have been analysed in 168 spindles and 19 tendon organs.

3. The first component requires a sufficiently strong shock to the muscle nerve, but neither tension nor extension of the muscle. It follows frequencies of the order of 130–200/sec in slack muscle and originates at the time of the muscle action potential, similarly in spindles and tendon organs. It is held to be an ephaptic stimulation of sensory terminals or nerve fibres.

4. The second group of early spikes at the foot of the contraction is conveniently studied in flexors because in them the ephaptic component is rare. About 40% of the flexor spindles discharge such spikes. In extensors they are preceded by ephaptic spikes.

5. These spikes are activated by fibres conducting at α velocity and do not depend upon tension but upon the phasic sensitivity of the spindles as determined by γ bias or equivalent extension of the equatorial region by pull on the muscle.

6. Flaxedil removes them before it removes γ activity and even in advance of the last remnant of extrafusal contraction, but re-activation follows after tetani to the muscle nerve.

7. The second group of early spikes is identified with the similarly located early spikes elicitable from various central stations (Granit, Pompeiano & Waltman, 1959).

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J. Physiol. (1959) 147, 419–42

THE EFFECT OF ADENOSINE TRIPHOSPHATE ON THE INTRA-OCULAR PRESSURE OF RABBITS*

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(*Received* 20 *March* 1959)

Antidromic stimulation of peripheral sensory nerves is known to cause vasodilatation of the skin in the region supplied by the nerve. Recently Holton & Holton (1953) and P. Holton (1956) have shown that the vasodilator effects of antidromic stimulation of the greater auricular nerve resemble those produced by the intra-arterial injection of adenosine triphosphate (ATP 1–10 μg) in the rabbit ear.

Antidromic stimulation of the first division of the trigeminal nerve in the rabbit causes a contraction of the pupil (Bernard, 1858; Maurice, 1954), a vasodilatation of the ocular vessels, an increased capillary permeability with the formation of a plasmoid aqueous and a rise in intra-ocular pressure (Perkins, 1954). In the course of investigations into these effects ATP has been injected intra-arterially, and into the anterior chamber of the rabbit's eye, to determine whether such injections could initiate the changes produced by antidromic stimulation of the trigeminal nerve.

METHODS

Adult rabbits were anaesthetized with urethane 1·75 g/kg intravenously. Recordings of intra-ocular pressure bilaterally and of the blood pressure were made with rubber-membrane manometers. A full description of the apparatus has been published (Perkins, 1957). The intra-arterial injections were made either into the common carotid artery through a T-shaped cannula with a rubber cap over the side arm (15 animals) or by retrograde injections into the facial artery (6 animals).

Two methods were used for injecting into the anterior chamber. In the first method (4 animals) one of the needles (N in Fig. 1) used for recording the intra-ocular pressure was connected directly to a three-way tap (T_1). One arm of the tap was connected to the manometer (M) by polythene tubing and the third to a 1 ml. tuberculin syringe (S). For each injection the three-way tap (T_1) was first turned to connect the anterior chamber to the syringe, containing 0·1 ml. of test solution. By slowly withdrawing and advancing the plunger of the syringe for 30 sec the test substance was mixed with the aqueous humour. Tap T_1 was then turned to connect the anterior chamber with the manometer and tap T_2 was opened to the reservoir of saline (R) to restore the intra-ocular pressure to its initial pre-injection level; T_2 was then closed.

In the second method (5 animals) the same apparatus was used except that the needle (N) was connected directly to the manometer and the syringe connected to the anterior chamber via a fine needle (35 gauge) and a two-way tap. During injections of 0·05 ml. of the test substances T_2 was opened to prevent an artificial rise in intra-ocular pressure due to the injection of fluid into the eye.

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* Part of a thesis for the degree of Ph.D., University of London.

Solutions of the Na salt of ATP were made up freshly for each experiment from a commercial preparation (Light and Co.). For injections into the anterior chamber solutions of the ATP Na were used in concentrations of 1 mg/ml. or 1 mg/0·5 ml. For the intra-arterial injections the ATP Na was derived from the more stable Ba salt (Light) and was prepared freshly for each experiment as follows. ATP Ba 30 mg was dissolved in 0·2 ml. N-HCl and the volume made up to 1·0 ml. with distilled water. Three drops of a saturated solution of Na_2SO_4 were added and the whole centrifuged and the supernatant fluid removed. The deposit remaining was washed with 1·0 ml. of distilled water and centrifuged again. The supernatants were pooled and neutralized with 0·1 N-NaOH to pH 6·7. For the control injections, $BaCl_2$ 30 mg was treated in the same manner.

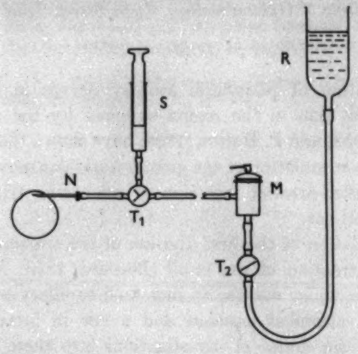

Fig. 1. Diagram of apparatus for injection into the anterior chamber of rabbit eye. N, hypodermic needle; S, 1 ml. tuberculin syringe; T_1, 3-way tap; M, rubber-membrane manometer; T_2, 2-way tap; R, reservoir of saline.

RESULTS

Intra-arterial injections

Injections of 1 ml. of saline intra-arterially did not produce any significant changes in intra-ocular pressure (I.O.P.). Injections of the control solution prepared from $BaCl_2$ produced a significant change (a rise) in one animal only.

ATP Na in doses of 1 μg intra-arterially caused a rise in I.O.P. from 5 to 10 cm saline in three out of four animals, without affecting the blood pressure; similar results were obtained with doses of 10 μg. 100 μg caused a rise in I.O.P. in nine out of eighteen animals and a fall of B.P. in nine animals. Higher doses caused a fall in B.P. in five out of six animals and a rise in I.O.P. in four animals.

Figure 2 shows the results obtained from one animal after the intra-arterial injection of saline and of 1, 10, 100 and 900 μg ATP. Small rises of I.O.P. in the ipsilateral eye followed each injection and a drop in blood pressure occurred after injection of 100 and 900 μg. The contralateral I.O.P. rose after the 900 μg injection. The results from all experiments are summarized in Tables 1 and 2.

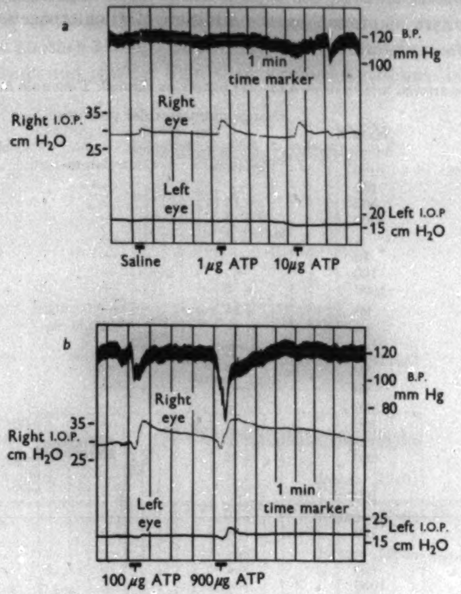

Fig. 2. *a*, recording of B.P. and I.O.P. of right and left eyes after injections of saline and of 1 μg and 10 μg ATP into right carotid artery. *b*, recording of B.P. and I.O.P. of right and left eyes after injections of 100 and 900 μg ATP into right carotid artery.

TABLE 1. Intra-arterial injections of ATP Na: injections into facial artery

Expt. No.	Amount injected (μg)	Change in intra-ocular pressure (cm saline)		Change in blood pressure (mm Hg)
		Ipsilateral	Contralateral	
1	100	− 4	—	—
2	100	− 2	—	—
	1000	—	—	− 60
3	1	+ 10	—	—
	10	+ 11	—	—
	100	+ 17	+ 2	—
4	1	+ 5	—	—
	10	+ 7	—	—
	100	+ 9	—	—
	900	+ 5	+ 5	− 40
5	1	+ 5	—	—
	10	—	—	—
	100	+ 27	+ 29	—

Examination of the aqueous at the end of each experiment produced a heavy precipitate of protein on the addition of trichloroacetic acid to the aqueous of the ipsilateral eye.

TABLE 2. Intra-arterial injections of ATP Na: injections through T-cannula in carotid artery

Expt. No.	Amount injected (μg)	Change in intra-ocular pressure (cm saline): Ipsilateral	Change in intra-ocular pressure (cm saline): Contralateral	Change in blood pressure (mm Hg)
1	100	—	—	-10
	500	+12	—	—
	1000	+ 6	—	—
2	1	—	—	—
	10	—	—	—
	100	—	—	- 5
	1000	+ 5	—	-40
3	90	+34	—	-40
4	90	+15	—	-60
5	90	+ 3	—	-54
	90	+ 8	+3	-50
6	90	—	—	-50
7	100 +	+ 5	—	—
	100	+ 7	—	—
	100 +	+ 6	—	—
	200	+19	—	—
8	($BaCl_2$ control)	—	—	—
	100	—	—	-20
9	($BaCl_2$ control)	+30	—	—
	100	+ 5	—	-20
10	($BaCl_2$ control)	—	—	—
	100	—	—	-20
	1000	—	—	-40
11	(Saline control)	—	—	—
	($BaCl_2$ control)	—	—	—
	100	+31	—	—
12	(Saline control)	—	—	—
	($BaCl_2$ control)	—	—	—
	100	- 7 followed by +22	—	-30
13	(Saline control)	—	—	—
	($BaCl_2$ control)	—	—	—
	100	—	—	—
14	(Saline control)	—	—	—
	($BaCl_2$ control)	—	—	—
	200	-4	—	-10
15	(Saline control)	—	—	—
	($BaCl_2$ control)	—	—	—
	1000	+2	+8	-20

Injections into the anterior chamber

Preliminary experiments showed that small doses of ATP Na produced little effect on I.O.P., but if 1 mg was injected a marked rise in I.O.P. followed in the majority of eyes. In one animal the injection of 0·1 ml. saline caused a marked rise in I.O.P., but in all nine other experiments control saline injections were without effect. Figure 3 is an example of a typical experiment.

In five animals out of ten a contraction of the pupil followed injections of ATP. In three of these the contraction was 0·5 mm or less, in one it was 1·5 mm and in another 3 mm. The pupillary contraction in these experiments was less constant and smaller than that obtained by stimulation of the trigeminal nerve.

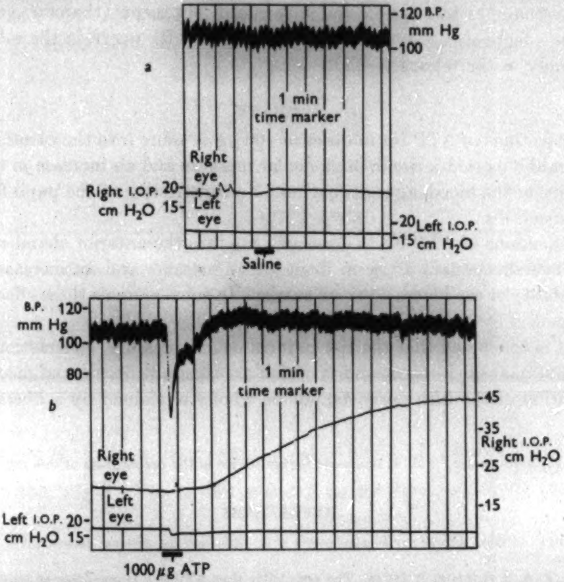

Fig. 3. *a*, recording of B.P. and I.O.P. of right and left eyes after injection of 0·1 ml. saline into right anterior chamber. *b*, recording of B.P. and I.O.P. of right and left eyes following injection of 1 mg ATP into right anterior chamber.

The aqueous humour from all the injected eyes showed a heavy precipitate of protein with trichloroacetic acid. In three animals protein was also present in the aqueous from the contralateral eye.

DISCUSSION

The main purpose of these experiments was to determine whether ATP given intra-arterially or directly into the anterior chamber of the rabbit's eye could initiate the phenomena produced by mechanical stimulation of the ophthalmic division of the trigeminal nerve. Although rises in intra-ocular pressure and increases in the permeability of the blood–aqueous barrier were obtained by such injections the effects were not as constant as those following stimulation of the trigeminal nerve. High and unphysiological doses were required to cause comparable rises in intra-ocular pressure by injections of ATP into the anterior chamber. Marked contraction of the pupil is a constant feature of stimulation of the fifth nerve in rabbits, but no pupillary changes followed the intra-arterial injections of ATP and those which did occur after injections into the anterior chamber were small and inconstant. It seems, therefore, unlikely that the effects on the eye of stimulation of the fifth nerve in the rabbit are due simply to the release of ATP.

SUMMARY

1. Injections of ATP Na in doses of 100 μg or more into the carotid artery of the rabbit caused a rise in intra-ocular pressure and an increase in the permeability of the blood–aqueous barrier. No constriction of the pupil followed these injections.

2. Injections of ATP Na in doses of 1 mg into the anterior chamber of the rabbit eye also caused a rise in intra-ocular pressure and an increase in the permeability of the blood–aqueous barrier. In some animals these effects were accompanied by a small contraction of the pupil.

3. It is concluded that the rise in intra-ocular pressure, contraction of the pupil and increase in permeability which are constant features of mechanical stimulation of the fifth nerve cannot be wholly explained by a liberation of ATP.

I should like to thank Dr N. A. Ambache for his advice in the preparation of this paper.

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J. Physiol. (1959) 147, 425–436

EFFECT OF ANTIGEN AND OCTYLAMINE ON MAST CELLS AND HISTAMINE CONTENT OF SENSITIZED GUINEA-PIG TISSUES

BY I. MOTA*

ERRATA

GOWENLOCK, A. H., MILLS, J. N. & THOMAS, S. (1959). *J. Physiol.* **146**, 133–141.

P. 133, l. 2: *for* '(Rosenbaum, Ferguson, Davis & Rossmeisl, 1952)' *read* '(for references see Rosenbaum, Ferguson, Davis & Rossmeisl, 1952)'.

BORN, G. V. R. & BRICKNELL, JANET (1959). *J. Physiol.* **147**, 153–161.

P. 156, Fig. 1. The letters *A* and *B* on the figure should be transposed.

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corresponding experiments with isolated tissue particles *in vitro*, under conditions in which oedema formation was excluded. A further aim of this work was to discover whether the various procedures used by Mongar & Schild (1957*a*, *b*, *c*) for inhibiting histamine release in anaphylaxis would also inhibit mast cell alterations induced by antigen. The action of octylamine on mast cells has also been studied. A brief report of this work has already been published (Mota, 1958).

METHODS

Guinea-pigs weighing about 300 g were sensitized with a 2% solution of egg albumin in 0·5% aqueous phenol, of which 1 ml. was administered subcutaneously and 1 ml. intraperitoneally. The animals were used from three weeks after sensitization. Evidence of sensitization was of the trigeminal nerve. High and unphysiological doses were required to cause comparable rises in intra-ocular pressure by injections of ATP into the anterior chamber. Marked contraction of the pupil is a constant feature of stimulation of the fifth nerve in rabbits, but no pupillary changes followed the intra-arterial injections of ATP and those which did occur after injections into the anterior chamber were small and inconstant. It seems, therefore, unlikely that the effects on the eye of stimulation of the fifth nerve in the rabbit are due simply to the release of ATP.

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SUMMARY

1. Injections of ATP Na in doses of 100 µg or more into the carotid artery

J. Physiol. **131**, 176–185.

Maurice, D. M. (1954). Constriction of the pupil in the rabbit by antidromic stimulation of the trigeminal nerve. *J. Physiol.* **123**, 45–46*P*.

Perkins, E. S. (1954). Experimental aspects of nervous control of the ocular circulation and intraocular pressure. *Acta XVII Concil. ophthal.* **2**, 1157–1163.

Perkins, E. S. (1957). Influence of the fifth cranial nerve on the intraocular pressure of the rabbit eye. *Brit. J. Ophthal.* **41**, 257–300.

J. Physiol. (1959) 147, 425–436

EFFECT OF ANTIGEN AND OCTYLAMINE ON MAST CELLS AND HISTAMINE CONTENT OF SENSITIZED GUINEA-PIG TISSUES

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(*Received* 25 *July* 1958)

It has been widely accepted that mammalian mast cells contain not only heparin but also histamine (Riley & West, 1953). In rats (Benditt, Wong, Arase & Roeper, 1955; Bhattacharya & Lewis, 1956) and in mice (Sjoerdsma, Waalkes & Weissbach, 1957) these cells may also contain 5-hydroxytryptamine. Histamine release induced by histamine liberators is usually associated with damage to mast cells (Riley, 1953; Mota, Beraldo & Junqueira, 1953; Fawcett, 1954).

Mast cell alterations induced by the antigen–antibody reaction in the guinea-pig were described by Mota & Vugman (1956). By making biopsies before and after the injection of antigen into sensitized guinea-pigs, these authors demonstrated a parallel decrease in the mast cell population and in the histamine content of the guinea-pig lung in anaphylaxis. This work was carried out in the intact guinea-pig and the occurrence of oedema during anaphylactic shock may have accounted for the reduction in the mast cell population and in the histamine content of the lung. It was decided therefore to carry out corresponding experiments with isolated tissue particles *in vitro*, under conditions in which oedema formation was excluded. A further aim of this work was to discover whether the various procedures used by Mongar & Schild (1957*a*, *b*, *c*) for inhibiting histamine release in anaphylaxis would also inhibit mast cell alterations induced by antigen. The action of octylamine on mast cells has also been studied. A brief report of this work has already been published (Mota, 1958).

METHODS

Guinea-pigs weighing about 300 g were sensitized with a 2% solution of egg albumin in 0·5% aqueous phenol, of which 1 ml. was administered subcutaneously and 1 ml. intraperitoneally. The animals were used from three weeks after sensitization. Evidence of sensitization was provided by testing whether the tissues released histamine in the presence of the specific antigen. Lung tissue was chopped with a McIlwain tissue chopper (McIlwain & Buddle, 1953) into rods of 0·8 × 0·8 mm cross-section. A series of uniform samples were prepared as described by Mongar & Schild (1953) and incubated at 37° C in 10 ml. beakers for 15 min with 0·9 ml. of the required concentration of inhibitor in Tyrode solution. The inhibitors used were: 0·001 M iodoacetate, 0·005 or 0·01 M phenol, and 0·01 % versene (sodium edetate). After this treatment either 0·1 ml. of Tyrode solution containing 1 mg of antigen or 0·2 mg of octylamine was added and the tissue incubated for a further period of 15 min.

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The mesentery was dissected out and cut into several small pieces which were dipped into cold Tyrode solution. Three or four of these pieces were used as a sample and incubated under conditions already described for the lung samples. The mesentery is a suitable preparation for the study of cytological details and the description of the mast cell alterations presented here is based chiefly on observations in the mesentery.

From each tissue several samples were prepared, some being used for mast cell counts and others for measuring either the amount of histamine released or the amount of histamine remaining inside the tissue. For the bioassay on the guinea-pig ileum of released histamine the solution was removed with a filter pipette from the lung particles or pieces of mesentery and diluted as required. The histamine content of the tissue was determined after heating the tissue in Tyrode solution in a boiling water-bath for 5 min. The histamine content of guinea-pig lungs was about 15 μg/g and that of mesentery 10 μg/g. For microscopical observation of the mast cells, the chopped lung tissue was fixed with a solution of lead subacetate in acidified alcohol as previously described (Mota & Vugman, 1956), and frozen sections 50 μ thick were stained with 0·1 % toluidine blue in acidified aqueous solution (pH about 4). The number of mast cells was determined in 100 randomly chosen microscopical fields with the aid of a square-ruled ocular micrometer, the magnification being × 250. The mesentery was fixed in the same fixative, examined as a whole-mount preparation and its mast cells counted in 30 microscopical fields, the magnification being × 80. In mounting the mesentery care was taken not to fold or stretch the tissue.

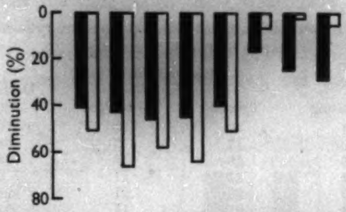
For mast cell counts on the ileum and uterus the fixative was injected under pressure into the lumen. The organs were then ligated at both ends and immersed in the fixative for 20–24 hr. Frozen sections 50 μ thick were prepared and stained with toluidine blue. Mast cell alterations were assessed either by calculating the percentage of partially degranulated cells, as compared with an untreated control, or by counting the total number of metachromatic granule-containing cells before and after treatment.

RESULTS

Effect of antigen–antibody reaction on mast cell morphology

In the guinea-pig lung the mast cells take the form of round, oval, elongated or spindle-shaped cells crowded with metachromatic granules. They are distributed in the walls of the alveoli, and are concentrated around the bronchioles (Pl. 1, fig. *A*). Their number (range 7–35 cells per field) varies in different individuals. In the mesentery the mast cells are found throughout the tissue, although they follow especially the blood vessels. They are uniform in their metachromasia and morphology, taking oval or elongated shapes (Pl. 2, fig. *E*). Their number varies (range 11–30 cells per field) in different animals.

When pieces of chopped lung tissue or mesentery from a sensitized guinea-pig are incubated at 37° C with antigen in Tyrode solution, damage to the mast cells occurs, as is shown by disappearance of their metachromatic material and a consequent reduction in the number of stainable mast cells (Pl. 1, fig. *B*). The antigen-induced alterations are characterized by a disappearance of the mast cell granules (degranulation) without any morphological evidence of their fate. No granules are found outside the cells and those remaining inside the cells keep their usual aspect (Pl. 2, fig. *F*). No such alterations of mast cells were seen when non-sensitized tissue was incubated with antigen. Even in tissues which have been in contact with antigen for only a few seconds the mast cells are partially deprived of their granules; no other recognizable alterations are seen. It seems that the granules are subjected to a quick dissolution or lysis either inside or outside the cells.



Text-fig. 1. Diminution of histamine content (■) and mast cell number (□) of chopped sensitized guinea-pig lung in anaphylaxis.

Effect of antigen–antibody reaction on mast cell number and histamine content of guinea-pig lung and mesentery

Lung

The effect of antigen on mast cell number and histamine content of chopped sensitized lung tissue in eight separate experiments is shown in Text-fig. 1. As a result of the anaphylactic reaction there is a diminution in both mast cell population and histamine content of the lung and there is a correlation between histamine release and mast cell reduction. Thus, in five experiments in which a substantial histamine release occurred there was also a substantial reduction in the mast cell number, whereas in the three experiments in which histamine release was less than 30% there was only a small decrease in mast cell number although many mast cells were degranulated.

Mesentery

Preliminary experiments showed that histamine is released by antigen from sensitized mesentery, although the amount released is usually too small to permit an accurate quantitative assay. On the other hand, the release of histamine in the control experiments was negligible. When sensitized mesentery is incubated with antigen the number of cells which can be recognized as mast cells becomes reduced. The results of incubation of pieces of mesentery with antigen are shown in Table 1. When these results were analysed by the t test the difference was found to be significant ($P < 0{\cdot}005$). In those cases in which there was no decrease in the mast cell number, qualitative alterations represented by partial degranulation of the mast cells were nevertheless seen, the number of degranulated mast cells in these cases varying from 15 to 68%.

Effects of inhibitors of anaphylaxis on mast cell alterations induced by antigen–antibody reaction

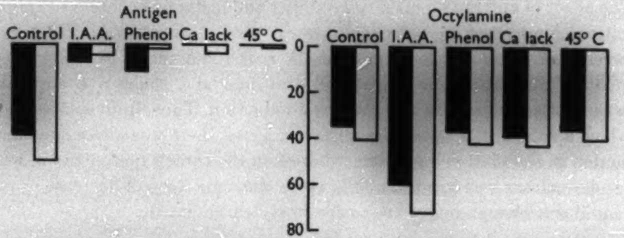
Experiments with lung tissue

The experiments with lung tissue are summarized in Text-fig. 2.

TABLE 1. Mast cell counts* in sensitized guinea-pig mesentery incubated with Tyrode solution or Tyrode plus antigen

	Mast cells in		
Guinea-pig	Tyrode	T + antigen	Decrease (%)
1	30	25	16
2	17	7	58
3	26	16	38
4	21	21	0
5	27	21	22
6	27	11	59
7	17	3	82
8	28	28	0
9	20	10	50
10	18	6	66
11	11	11	0

* Means of 30 microscopical fields at a magnification of × 80.



Text-fig. 2. Effect of various inhibitors on histamine release (■) and mast cell decrease (☐) in chopped sensitized guinea-pig lung by antigen or by octylamine. Figures represent percentage diminution.

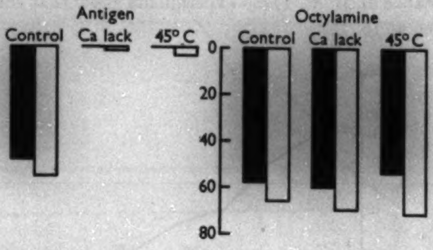
Iodoacetate or phenol. When sensitized lung particles were incubated with antigen in the presence of iodoacetate (0·001 M) or phenol (0·005 M), the damage to mast cells and the histamine release by antigen were greatly reduced.

Calcium lack. When the sensitized lung particles were incubated in calcium-free Tyrode solution containing versene there was a total inhibition of mast cell damage and of histamine release by antigen, but when calcium ions were added subsequently both histamine release and mast cell disruption occurred.

Previous heating of the tissue. When sensitized lung particles were kept at 45° C for 15 min and subsequently incubated with antigen at 37° C there was no mast cell damage or histamine release. Heating at 45° C itself produced no visible mast cell damage.

Experiments with mesentery

The effects of previous heating of this tissue at 45° C for 15 min and of the absence of calcium on mast cell destruction and histamine release are shown in Text-fig. 3. As in the lung, both these treatments inhibited mast cell damage by antigen. When iodoacetate or phenol was used the amount of histamine released from the mesentery by antigen was too small to be detected and no decrease in mast cell number occurred, but qualitative alterations were present.



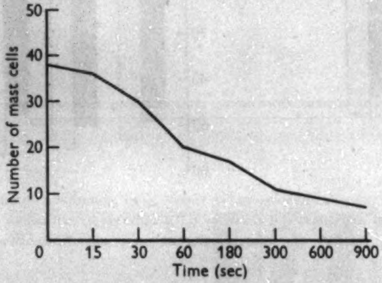
Text-fig. 3. Effect of previous heating of the tissue to 45° C and the absence of calcium on histamine release (■) and mast cell decrease (□) induced by antigen or by octylamine in sensitized guinea-pig mesentery. Figures represent percentage diminution.

Effect of phenol on desensitization

Some experiments were then carried out to see if phenol would inhibit desensitization as well as mast cell destruction. A preliminary test was made to see whether phenol denatures antibody. Sensitized mesentery was incubated for 15 min with 10 mM phenol in Tyrode solution and then transferred to Tyrode solution containing no phenol. Addition of antigen now produced the usual degranulation of mast cells, showing that antibody was not denatured. The next test was whether phenol prevents desensitization. When antigen was added in the presence of 10 mM phenol there was no mast cell damage, as has already been shown. If now the mesentery was washed in 10 mM phenol to remove antigen, then in Tyrode solution to remove phenol, and a second dose was added, this also failed to produce mast cell damage. Thus the mast cell had been desensitized without becoming degranulated.

Effect of low temperature on mast cell destruction by antigen–antibody reaction

To test the effect of low temperature, pieces of sensitized mesentery were incubated with antigen at 15° C for 15 min; some of the pieces were then immediately transferred to fixative and others to antigen solution at 37° C, kept there for 15 min, and fixed afterwards. Microscopical observation of the tissue showed that mast cell degranulation was absent in the pieces of mesentery that had been fixed immediately after contact with antigen at 15° C, but present in those that were fixed after warming to 37° C. However, the diminution in mast cell number in the pieces of mesentery that had been in contact with antigen at 15° C and were subsequently brought to 37° C was smaller than in control pieces from the same mesentery in which antigen was added in the first instance at 37° C. These experiments show that some desensitization occurs even at 15° C.



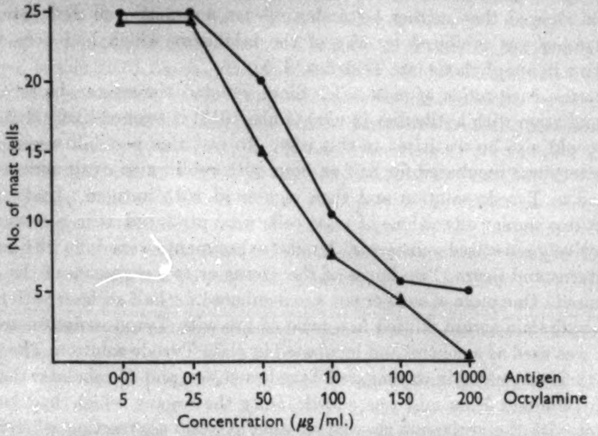
Text-fig. 4. Effect of time of incubation with antigen on the number of mast cells in sensitized guinea-pig mesentery. Note irregular abscissa scale.

Effect of time of incubation with antigen on mast cell degranulation and histamine release

Pieces of sensitized mesentery were incubated with antigen for periods of 15 sec up to 15 min. The results are shown in Text-fig. 4. A significant decrease (50–70 %) in mast cell number was detected as early as 1 min after incubation and degranulation was seen to occur after about 15 sec incubation. To test whether histamine release also occurs within 15 sec, pieces of mesentery were incubated for 15 sec in 1 ml. of Tyrode solution and afterwards transferred to 1 ml. of a 0·1 % solution of antigen in Tyrode for the same period. Both control and antigen solutions in which the mesentery was incubated were then tested for histamine content. In each of seven experiments a detectable amount of histamine was released after 15 sec contact of the tissue with antigen.

Effect of concentration of antigen on mast cells

To see if mast cell alterations increased with the concentration of antigen, pieces of sensitized mesentery were incubated with different concentrations of antigen for 15 min at 37° C and afterwards fixed. The results of these experiments are summarized in Text-fig. 5, which shows that the decrease in mast cell number is related to the concentration of antigen. Antigen is active in very low concentrations of the order of 10^{-6}.



Text-fig. 5. Effect of different concentrations of antigen (●—●) and octylamine (▲—▲) on mast cell number in sensitized mesentery. Semi-log. scale for antigen.

Comparison with octylamine effect

When pieces of guinea-pig lung or mesentery are incubated with octylamine in Tyrode solution at 37° C, damage to the mast cells occurs, as is shown by reduction in the number of these cells. As with antigen–antibody reaction this reduction is proportional to the concentration of the substance and in the concentrations of 0·2 mg/ml. octylamine is sometimes more effective than antigen (see Text-fig. 5). Thus both the antigen–antibody reaction and octylamine produce conspicuous alterations of the same cell. However, cytologically the alterations induced by octylamine are different from those induced by antigen. The first visible change induced by octylamine is an apparent loss of the granule limit, as if a diffusion of the metachromatic material from the granules had taken place (Pl. 2, fig. *G*). When this phenomenon progresses the cell looks like a metachromatic blot, in which some basophilic granules can sometimes be noticed. In the final stages these metachromatic blots present an irregular contour and a faint metachromasia and the cell is apparently in a state of dissolution. It is interesting that the morphological difference between the mast cell damage induced by the antigen–antibody reaction and by octylamine is a very sharp one. Thus, while the antigen-induced mast cell alterations are characterized by a total or partial loss of the granules without fusion of the metachromatic material, the octylamine-induced mast cell alterations are characterized by an apparent fusion of the metachromatic material without any loss of granules. Furthermore, when the effect of the several inhibitors of anaphylaxis on mast cell destruction by octylamine was tried, the results showed that neither histamine release nor mast cell destruction by octylamine was inhibited by any of the inhibitors which had been found effective in anaphylaxis (see Text-figs. 2, 3).

Passive sensitization of mast cells. Since isolated tissues can be sensitized by incubation with antibodies *in vitro* (Dale, 1913) it seemed likely that mast cells could also be sensitized in this way. To test this possibility guinea-pig mesentery was incubated for half an hour with rabbit anti-ovalbumin serum, washed in Tyrode solution and then incubated with antigen. Under these conditions similar alterations of mast cells were produced as in mesentery of the actively sensitized guinea-pig. Similar experiments were done with guinea-pig uterus and ileum. Two horns of the uterus or two segments of the ileum were used. One piece of each organ was incubated for half an hour with rabbit anti-ovalbumin serum diluted in a ratio of 1:5 with Tyrode solution and the other was used as a control and incubated in plain Tyrode solution. The pieces were then suspended in oxygenated Tyrode solution and 15 min later the antigen (ovalbumin 1 mg/ml.) was added. Only the organs which had been in contact with the antiserum showed an anaphylactic contraction whereas the controls gave no reaction. After 30 min contact with antigen the pieces were removed and fixed. On microscopical inspection both sensitized uterus and ileum showed typical degranulation of the mast cells present in the mucosa and amongst the muscle fibres, whilst the control pieces showed no visible mast cell alterations (Pl. 1, figs. *C* and *D*).

DISCUSSION

The mast cell alterations produced *in vitro* by the antigen–antibody reaction are identical with those previously described in the intact animal; both conditions are characterized by a disappearance of mast cell granules. It is known that mast cell granules are of a lipidic-proteic nature (Hedborn & Snellman, 1955) and their quick disappearance induced by antigen in sensitized tissues may be mediated through lysis induced by activation of an enzyme as suggested by Rocha e Silva (1944). This is also suggested by the dependence of this phenomenon on temperature and its inhibition by metabolic inhibitors.

The morphological alterations induced by octylamine suggest that this base combines directly with the metachromatic material in the granules, and in so doing produces a complete disorganization of the granular structure with subsequent histamine release. A similar mechanism of action was postulated by MacIntosh (1956), based on experiments with intracellular particles.

It is interesting to emphasize here that the morphological difference between the mast cell damage, induced by the antigen–antibody reaction and by octylamine, might also reflect a difference in the mechanism of action of these agents on the mast cell, although both produce histamine release. This agrees with the fact that iodoacetate, phenol, calcium lack or heating to 45° C, although producing a partial or total inhibition of mast cell destruction by antigen in sensitized tissues, do not block the mast cell destruction by octylamine. These results are also in agreement with the conclusion by Mongar & Schild (1957*a*, *b*) that in the guinea-pig the histamine-releasing mechanism of the antigen–antibody reaction is different from that of octylamine. Nevertheless, the fact that both antigen–antibody reaction and octylamine produce alterations of mast cells shows that at least part of the histamine released by these two agents originates from the same source. Other histamine releasers may act on the guinea-pig mast cells in a similar way to octylamine; thus we have observed that compound 48/80 (Baltzly, Buck, de Beer & Webb, 1949) *in vitro* induces mast cell alterations similar to those produced by octylamine, although this compound was effective only in much higher concentration than octylamine.

The very short time in which detectable morphological alterations of mast cells and histamine release can be produced in the mesentery can be explained by the thinness of this tissue and the high velocity of the antigen–antibody reaction (Mayer & Heidelberger, 1942). The finding that in the presence of phenol desensitization may occur without mast cell degranulation suggests that the antigen–antibody reaction itself is not responsible for degranulation but that this is a consequence of some later reaction. This is also indicated by the fact that a partial desensitization occurs when sensitized tissue contacts antigen in the cold.

It was postulated by Schild (1956) that when a mast cell disintegrates during the anaphylactic reaction it creates a histamine gradient in its surroundings, and that the degree of muscular contraction will then depend on the number of plain muscle cells reached by effective concentrations of histamine. Our results, which suggest that mast cells release histamine in anaphylaxis, together with the finding that in the guinea-pig lung, ileum and uterus these cells are in close proximity to the smooth muscle fibres, fit in well with this hypothesis. This concept implies that the 'intrinsic histamine' of Dale might originate from the mast cells, although it does not exclude the possibility that some of the released histamine comes from the muscle cells themselves. In a wider sense 'intrinsic histamine' could be defined as histamine liberated from, or in close proximity to, the responsive structure so that the distinction between extrinsic and intrinsic histamine would not be a sharp one.

In relation to the hypothesis that histamine released in anaphylaxis may originate from mast cells, it is interesting to observe that mast cells are in close association with the responsive structures in the so-called shock organ, as, for example, in bronchial muscle of the guinea-pig lung and in the muscular layer of the veins in dog liver (Mota, Ferri & Junqueira, 1956).

The correlation found between mast cell destruction and histamine release suggests that histamine released in anaphylaxis originates from mast cells, although it does not prove that these cells are the only source of released histamine. This correlation, together with the finding of other workers (Armitage, Herxheimer & Rose, 1952; Weissbach, Waalkes & Udenfriend, 1957) that substances other than histamine do not fulfil a major role in anaphylaxis in the guinea-pig, indicates that mast cells play a very important part in the anaphylactic reaction of this species. The finding that mast cell alterations occur in anaphylaxis in the dog (Jaques & Waters, 1941), the rat (Mota, 1957), and the mouse (Carter, Higginbotham & Dougherty, 1957) and in active and passive anaphylaxis in the guinea-pig, suggests that these cells might be particularly concerned with the uptake of antibodies: this problem is being investigated.

SUMMARY

1. Antigen (ovalbumin) produces mast cell alterations in sensitized guinea-pig lung, ileum, uterus and mesentery. These alterations are characterized by a disappearance of mast cell granules and a reduction in the total number of these cells.

2. The mast cell alterations occur simultaneously with histamine release and both processes are inhibited by iodoacetate, phenol, calcium lack, previous heating of the tissue to 45° C and cooling to 15° C.

3. The mast cell damage, induced by antigen in sensitized tissues, does not appear to be a direct result of the antigen–antibody union but rather a consequence of some later reaction.

4. Octylamine produces mast cell alterations that are morphologically different from those induced by antigen, and are characterized by an apparent diffusion of the metachromatic material from the granules.

5. The mast cell alterations induced by octylamine are also accompanied by histamine release but they are not inhibited by the various inhibitors of anaphylaxis.

6. It is suggested that most of the histamine released in anaphylaxis comes from the mast cells and that these cells may be concerned with the uptake of antibodies.

The author is greatly indebted to Dr H. O. Schild for his valuable advice and helpful suggestions during this work.

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EXPLANATION OF PLATES

PLATE 1

(Toluidine blue stain.)

A. Mast cells in sensitized guinea-pig lung incubated with Tyrode solution, approx. ×110. Note numerous mast cells distributed through the pulmonary tissue and concentrated around the bronchioles.

B. Mast cells in sensitized guinea-pig lung after contact with ovalbumin, approx. ×110. Note diminution in mast cell number.

C. Mast cells in non-sensitized guinea-pig ileum after contact with ovalbumin, approx. ×160. Note mast cells crowded with granules in the mucosa and muscular layer.

D. Mast cells in sensitized guinea-pig ileum after contact with ovalbumin, approx. ×160. Note degranulated mast cells in the mucosa and in the muscle layer.

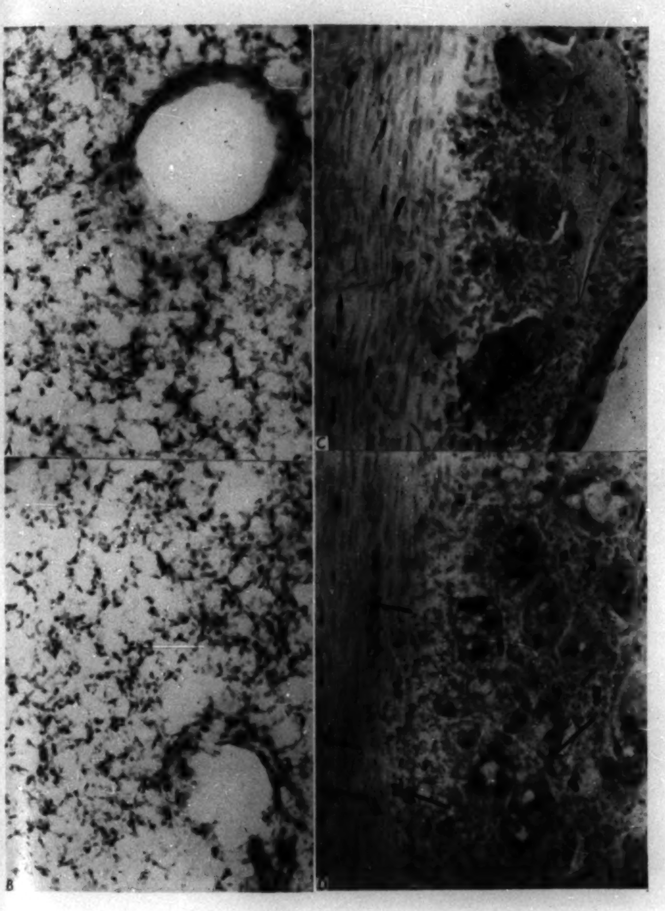
PLATE 2

(Toluidine blue stain, approx. ×1000.)

E. Mast cell in sensitized guinea-pig mesentery incubated with Tyrode solution. Note cell crowded with granules.

F. Mast cell in sensitized guinea-pig mesentery incubated with ovalbumin. Note that the cell has lost most of its granules.

G. Mast cell in sensitized guinea-pig mesentery incubated with octylamine. Note the aspect of fusion of the metachromatic material.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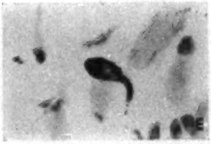
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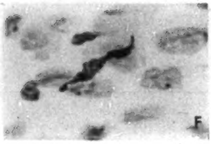
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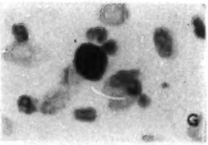
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J. Physiol. (1959) 147, 437–449

THE DISTRIBUTION AND FATE OF RADIOACTIVE HISTAMINE IN THE RAT

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It is universally accepted that histamine, whether of endogenous or exogenous origin, is disposed of by the two processes of enzymic destruction and of excretion through the renal tract. There is, however, little information about the way in which exogenous histamine is brought into contact with its specific enzymes. The rapid clearance of histamine from the blood after its intravenous injection (Rose & Browne, 1938) suggests that the uptake of histamine by the tissues may be a method for its immediate disposal from the blood, related to, and as important as, its subsequent metabolic destruction and renal excretion. The details and mechanisms of this process are still unexplained. The purpose of the present experiments was to investigate this process by means of radioactive histamine, and to examine the fate, distribution and transfer through the tissues of exogenous histamine introduced into the circulation of the living organism.

Rose & Browne (1938) suggested that a powerful mechanism existed for the uptake of histamine into the tissues from the circulating blood of the rat, but their results have been criticized on account of the large doses of histamine which they used. Such doses may cause disturbances in the circulation after intravenous injection. In the present experiments, therefore, a relatively small dose of histamine was used for injections so that effects due to circulatory changes could be excluded.

METHODS

Albino male rats of the Wistar strain weighing 125–150 g were used in all the experiments. After the intravenous injection of radioactive histamine, blood was removed at intervals of 15, 20 and 40 sec through a fine cannula inserted into the carotid artery, and at intervals from 1 to 240 min by puncturing the retro-orbital plexus, as described by Halpern & Pacaud (1951). The brain, liver, spleen, lungs, heart, kidneys, alimentary canal, striated muscle, and skin were removed for extraction and assay of histamine at intervals varying from 5 to 240 min after the injection. Urine was collected for histamine assay by puncture of the bladder after ligation of the penis to ensure that none was lost.

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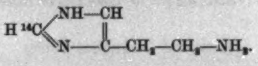
The experiments were performed on intact animals on which an effective nephrectomy had been performed by ligation of the renal pedicles. To avoid the effect of surgical shock, silk ligatures were placed loosely round the renal pedicles in a preliminary operation, performed under ether anaesthesia, and the ends of the ligatures were brought to the surface of the skin in the back through the operation wound. Four or five days after the animals had recovered from the operation, the ligatures were tightened and secured round the renal vessels. After each experiment had been completed, post-mortem examination was performed, and it was invariably confirmed that the renal pedicles had been effectively ligated, and that the kidneys had been completely excluded from the circulatory system.

Extraction and assay of radioactive ^{14}C *histamine.* Histamine was used in the form of the bihydrochloride, marked with ^{14}C in the imidazole nucleus in position 2:



The radioactive histamine was prepared from potassium thiocyanate K-^{14}CNS by the method of Frazer & Raphael (1952) at the radiochemical centre, Amersham, England. The specific activities were 4·176 mc/mM and 1·48 mc/mM for the two samples which were used. The first sample was diluted with non-isotopic histamine in the following proportions: crystallized histamine bihydrochloride ^{14}C 96·2 parts + 3·8 parts histamine bihydrochloride (Roche). The second sample was injected undiluted.

A known volume of blood plasma was spread in a thin layer on a glass slide 22 mm in diameter and the radioactivity was measured in a Geiger–Müller counter. The radioactivity of the erythrocytes was measured in the same way after the cells had been haemolysed by diluting them 1:4 with distilled water and then exposing them on the glass slide in the counter.

The rats were killed by bleeding them to death. Immediately afterwards 300–400 mg of each tissue was cut in a freezing microtome into sections of 1 μ and placed in an aqueous solution of Tween 80 1/1000. The tissue suspension was spread in a thin uniform layer on a round glass slide of 22 mm diameter, which corresponded to the diameter of the window in the Geiger–Müller counter. The slide was mounted in an aluminium cup and was placed at the opening of the counter where the maximum exposure for measurement of the β radiation was obtained. The actual quantity of ^{14}C in the tissues was calculated by using a layer of tissue of uniform thickness for absorption of the β rays. The depth of the layer was established by adding known quantities of radioactive histamine to samples of the tissues of various depths. A tissue layer of 50μ was finally selected as a suitable depth for giving reproducible results in all the tissues. Under these conditions, and with the apparatus which was used (Tracerlab), 10% of the radioactive ^{14}C was recovered. In order to make comparisons between the values for the biologically active histamine and the radioactive ^{14}C in the tissues, the latter has been expressed in terms of the chemically unmodified histamine bihydrochloride in the results, and is referred to as 'radioactive histamine'.

Extraction and assay of biologically active histamine. The 1μ tissue slices were immediately placed in N-HCl. After boiling, the histamine was extracted by Code's method (Code, 1937) and was assayed on the atropinized guinea-pig ileum. The results are expressed in terms of histamine bihydrochloride.

RESULTS

The radioactive and biologically active histamine in the blood and tissues were measured in parallel. The values for the biologically active histamine normally present in the lungs, liver, striated muscle and kidney were measured in twelve control animals. The mean values were used in order to calculate the quantity of exogenous biologically active histamine fixed by the tissues in the experimental animals. The quantity of exogenous biologically active histamine fixed by the tissues was found by obtaining the difference between the quantity in the tissues of the experimental animals after the injection of the exogenous histamine, and the quantity present in the tissues of the control animals. By observing the changes in the values for the radioactive histamine and for the biologically active histamine at intervals after the injection of the exogenous histamine, the extent of its metabolism in different organs was calculated. The experiments were performed on normal animals and on animals whose kidneys had been excluded from the circulation, so as to assess the importance of the kidneys in the simultaneous processes of destructive oxidation and renal excretion of histamine in comparison with its destructive oxidation by the other tissues of the body.

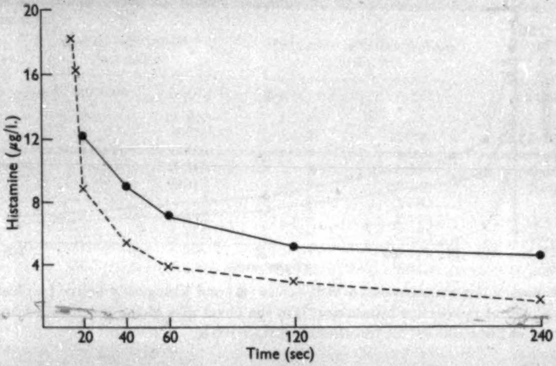


Fig. 1. Changes in the concentration of radioactive (●) and biologically active (×) histamine in plasma of the rat during the first 4 min after the intravenous injection of histamine ^{14}C bihydrochloride 500 μg/100 g. Radioactive histamine is calculated from the values for radioactive ^{14}C, and is expressed, like the biologically active histamine, as histamine bihydrochloride μg/l.

Normal animals

Plasma histamine. Intravenously injected histamine leaves the circulating blood rapidly. The plasma histamine measured at 15, 20 and 40 sec, and at intervals from 1 to 240 min after the intravenous injection of radioactive histamine 500 μg/100 g, is shown in Figs. 1 and 2. During the first minute the concentration of both radioactive and biologically active histamine diminished quickly, subsequently their rates of disappearance became slower, and, depending on whether the biologically active or radioactive form was being examined, the fall continued for about 15 min or several hours.

After the injection, when the histamine had become completely mixed with the extracellular fluid, the volume of which is about 20 ml. in rats weighing 100 g, the histamine concentration would have been 25,000 μg/l. However, 15 sec after the injection the plasma only contained 18,300 μg/l., and after 1 min it had fallen to 3810 μg/l.; that is, about 85 % of the biologically active histamine had disappeared. Its value had returned to the normal level 30 min after the injection. The concentration of radioactive histamine diminished more slowly than that of the biologically active form. This difference was already evident during the first minute, but it became more pronounced later on. Thirty minutes after the injection the plasma still contained 2010 μg/l.; that is, about 8 % of the theoretical concentration at zero time. It was not until 4 hr after the injection that the radioactive histamine had almost entirely disappeared.

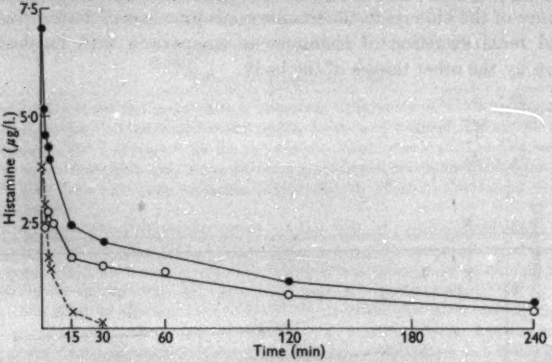


Fig. 2. Changes in the concentration of radioactive (●) and biologically active (×) histamine in plasma, and of radioactive histamine (○) in the blood cells of the rat during 4 hr after the injection of histamine ^{14}C bihydrochloride 500 μg/100 g.

The difference between the values for the radioactive and biologically active histamine corresponds to the quantity of histamine which contains the imidazole nucleus. By calculating the fraction

$$\frac{\text{biologically active histamine}}{\text{radioactive histamine}}$$

the proportions of the active and inactive forms remaining in the circulation can be estimated, and the rate of the histamine metabolism can be measured. This has been done in Table 1, and the rate of metabolism of the biologically active histamine as expressed by its decline from unity is shown.

Red blood cell histamine. Exogenous histamine penetrated rapidly into the cellular fraction of the blood; afterwards its concentration in the red cells declined as in the plasma. The concentration in the cells was generally lower than in the plasma, but at the end of 4 hr the values for the radioactive histamine in the cells and plasma had diminished to almost the same low level (Fig. 2).

Tissue histamine. Rose & Browne (1938) suggested that a powerful mechanism existed for the uptake of histamine into the tissues from the circulating blood of the rat, but their results have been criticized on account of the large doses of histamine which they used. Such doses may cause disturbances in the circulation after intravenous injection. The raised values for the tissue histamine found in the present experiments following the injection of a relatively small dose of histamine confirm their observations and show that such effects occur in the absence of any circulatory changes induced by the histamine. The disappearance of the histamine from the circulation appeared to be too fast wholly to be explained by its enzymic destruction or urinary excretion, and so the rate of uptake of both forms of histamine by various tissues was measured.

TABLE 1. Changes in radioactive and biologically active histamine in plasma, and in the fraction

$$\frac{\text{biologically active histamine}}{\text{radioactive histamine}}$$

of rats after intravenous injectionof histamine ^{14}C bihydrochloride 500 µg/100 g. Injection at time zero

Time after injection	Biologically active histamine		Histamine calculated from plasma ^{14}C		Biologically active histamine / Radioactive histamine
	No. of animals	(µg/l.)	No. of animals	(µg/l.)	
15 sec	9	18·300	—	—	—
20	9	8·870	3	12·080	0·735
40	9	5·410	3	8·900	0·605
1 min	9	3·810	9	7·050	0·540
2	7	2·910	9	5·150	0·550
3	—	—	6	4·560	—
4	7	1·685	6	3·770	0·450
5	28	1·404	6	3·470	0·400
15	21	0·360	3	2·420	0·150
30	21	0·100	3	2·010	0·047
60	—	—	—	—	—
120	—	—	3	1·080	—
180	—	—	—	—	—
240	—	—	3	0·560	—

After the injection of the radioactive histamine it rapidly diffused into the different tissues, but the quantity taken up by them varied considerably. The organs which took up most histamine were the kidneys, striated muscle, liver, skin, and ileum; elsewhere the concentrations were considerably less. Biological assay was only performed in the tissues in which it was thought that the uptake or metabolism would be greatest. On account of the high normal content of the skin, the contribution made to it by the injection of exogenous histamine was relatively small and insufficiently accurate to provide figures of any value. Five minutes after the injection, 57 % of the total quantity which was taken up by the kidney, as indicated by the uptake of the radioactive form, was found to be biologically active. The biologically active histamine found in liver and striated muscles at this time was only 41 % of the total uptake by these organs. The relative quantities of both forms of histamine found in the different tissues at various periods after its administration are shown in Fig. 3.

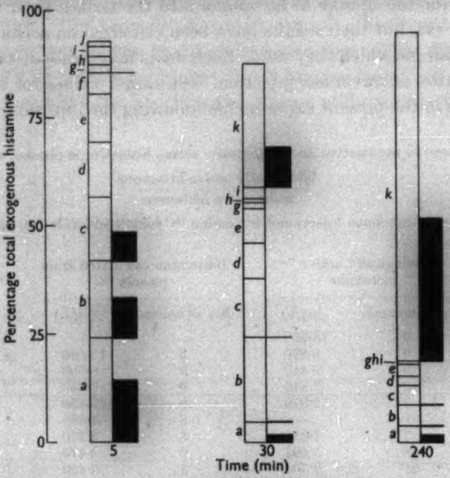


Fig. 3. Radioactive (☐) and biologically active (■) histamine found in blood, urine and different organs, 5, 30 and 240 min after the intravenous injection of histamine ^{14}C bihydrochloride 500 μg/100 g, expressed as percentages of the injected histamine. The figures indicate the values per whole organ. The skin, striated muscle and blood have been taken as 15, 45 and 8 % of the body weight, respectively. *a*, kidney; *b*, striated muscle (thigh); *c*, liver; *d*, abdominal skin; *e*, ileum (without contents); *f*, whole blood; *g*, lungs; *h*, myocardium; *i*, colon (without contents); *j*, spleen; *k*, urine.

The biologically active histamine diminished more rapidly than the radioactive histamine, and as the histamine decreased in the tissues it increased in the urine. The disappearance of the biologically active histamine in the tissues results therefore partly from urinary excretion, partly from enzymic destruction.

Table 2 shows the uptake of histamine calculated not for the whole organs but per gram of tissue. It can be seen that 5 min after the injection of radioactive histamine the kidney has absorbed the largest quantity, namely 146 μg/g. The heart, liver and lung follow in descending order, each having absorbed about one tenth as much as the kidney; the striated muscle contained the lowest concentration. The concentrations of biologically active histamine in the different tissues followed the same pattern as the quantities taken up by the individual organs shown in Fig. 3. The results given in Table 2 show that the kidney has an extremely potent histamine-extracting capacity, which has also been observed by Lindell & Schayer (1958) for the dog's kidney.

TABLE 2. Content of radioactive histamine in the organs. Values represent the mean of three animals and are expressed in μg/g of the fresh organ

Tissue	5 min	30 min	240 min
Kidney	146·5	23·9	15·3
Heart	21·0	8·6	1·3
Liver	16·9	17·1	5·4
Lung	10·6	7·8	1·4
Ileum	9·8	6·7	3·5
Colon	5·6	5·1	1·6
Spleen	5·5	4·8	2·3
Stomach	5·4	2·1	1·9
Skin	4·2	2·9	0·7
Muscle	2·0	2·4	0·6

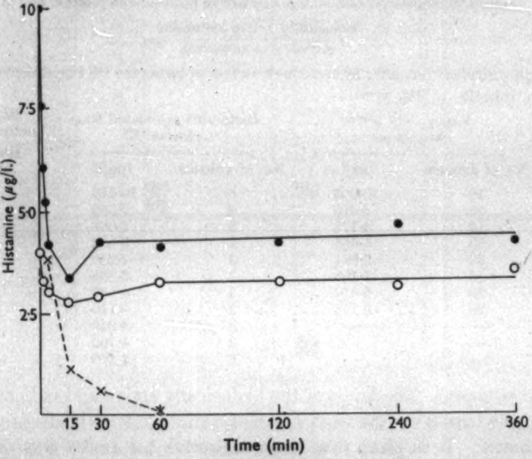


Fig. 4. Changes in the concentration of radioactive (●) and biologically active (×) histamine in the plasma, and of radioactive histamine (○) in the blood cells, of the rat with kidneys excluded from the circulation during 4 hr after the injection of histamine ^{14}C bihydrochloride 500 μg/100 g.

Animals with kidneys excluded from the circulation

The predominating effect of the kidney in controlling the transfer of histamine in the living animal made it desirable to study the histamine metabolism in animals in which the renal vessels had been ligated.

Blood histamine. The values for both forms of histamine in the blood of nephrectomized animals after an intravenous injection of radioactive histamine 500 μg/100 g are shown in Fig. 4 and Table 3. During the first minutes the rate of disappearance of the histamine from the circulation resembled that in normal animals; but later the histamine remained about 30% higher in the nephrectomized than in the normal animals. It is clear that the values for the biologically active histamine were considerably raised in the animals whose kidneys had been excluded from the circulation, and that its rate of disappearance was reduced in comparison with normal animals. The concentration of radioactive histamine in the plasma diminished during the first 30 min, but thereafter it did not change significantly for a period of several hours. As was the case in normal animals, the changes in the concentration of radioactive histamine in the blood cells were similar to those in the plasma, although the concentration in the cells remained about 2% less during the whole experiment.

Table 3. Changes in radioactive and biologically active histamine in plasma, and in the fraction

$$\frac{\text{biologically active histamine}}{\text{radioactive histamine}}$$

of nephrectomized rats after intravenous injection of histamine ^{14}C bihydrochloride 500 μg/100 g. Injection at time zero

Time after injection (min)	Biologically active histamine		Histamine calculated from plasma ^{14}C		$\frac{\text{Biologically active histamine}}{\text{Radioactive histamine}}$
	No. of animals	(μg/l.)	No. of animals	(μg/l.)	
1	10	10·000	6	10·110	0·99
2	10	6·615	6	7·586	0·88
3	10	4·775	3	6·066	0·80
4	10	4·685	3	5·250	0·89
5	30	3·924	4	4·240	0·92
15	20	1·100	3	3·360	0·33
30	20	0·617	3	4·160	0·15
60	20	0·145	3	4·140	0·04
120	—	—	3	4·240	—
180	—	—	3	4·700	—
240	—	—	3	4·260	—

Tissue histamine. The levels of the biologically active and radioactive histamine in the tissues are shown in Fig. 5 at various intervals after injection of the histamine. It is clear that the radioactive histamine was distributed throughout all the remaining organs when the kidneys were excluded from the circulation, and was not selectively concentrated in any of them. Most of the tissues took up some of the injected histamine; the uptake varied between 2 and 5% of the total according to the organ. An interesting observation made in these experiments on nephrectomized animals was that a transfer of radioactive histamine took place between the organs. Certain tissues such as the liver, ileum and heart absorbed the histamine initially at the expense of other tissues. Later they lost this store of histamine, which passed to the muscles and skin. The quantity of biologically active histamine stored in the organs decreased progressively following the injection, and the fraction

$$\frac{\text{biologically active histamine}}{\text{radioactive histamine}}$$

correspondingly diminished. This indicates that a progressive destruction of the biologically active histamine was occurring in the tissues.

Turnover of exogenous histamine in the tissues

After the administration of histamine marked with ^{14}C in the imidazole nucleus, information is obtained about the movement and fate of the radioactive histamine without knowing whether the molecule is still biologically active or has been broken down. Biological assay alone indicates whether the injected histamine is present in the blood and tissues in an unaltered form. The percentage of the injected histamine which has been metabolized at any instant in the tissues is indicated by the percentage of the radioactive histamine which is biologically inactive. This can be calculated from the following formula:

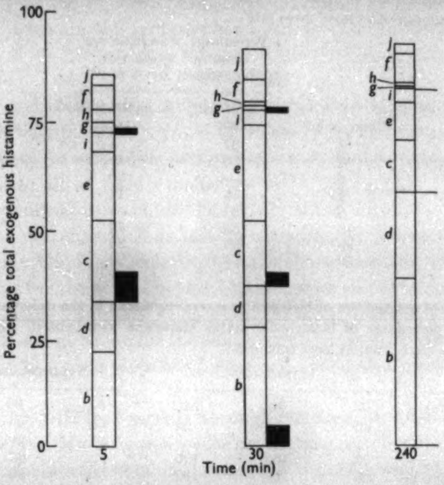


Fig. 5. Percentage of radioactive and of biologically active histamine found in the blood and different organs of nephrectomized rats after the intravenous injection of histamine ^{14}C bihydrochloride 500 $\mu g/100$ g. Conventions as in Fig. 3.

$$\frac{100-\left(\begin{matrix}\text{Total biologically active}\\ \text{histamine found}\end{matrix}-\begin{matrix}\text{Biologically active histamine}\\ \text{normally present}\end{matrix}\right)\times 100}{\text{Radioactive histamine}}$$

$$= \text{percentage of radioactive histamine metabolized.}$$

Figures indicating the rate at which the radioactive histamine in the plasma was metabolized are shown in Table 4. These figures demonstrate that the biologically active histamine disappeared rapidly, and that 5 min after its injection 60 % of the radioactive histamine had been transformed into a physiologically inactive form. In the nephrectomized animal the rate of transformation appeared to be reduced, although a larger number of observations will be necessary definitely to confirm this.

TABLE 4. Changes in the value of the biologically active histamine calculated as a percentage of the radioactive histamine in the plasma of normal and nephrectomized rats after the injection of histamine ^{14}C bihydrochloride 500 μg/100 g at zero time. Each observation represents the mean of at least three rats

	Percentage of radioactive histamine which was biologically inactive in	
Time (min)	Normal rats	Nephrectomized rats
1	37	1
2	36	12
3	—	20
4	56	11
5	60	8
15	88	73
30	95	85
60	—	97

TABLE 5. The rate of metabolism of biologically active histamine in the different tissues: values expressed as percentage of biologically active histamine metabolized. Each observation represents the mean from at least three rats

	Control rats			Nephrectomized rats		
Time (min)	5	30	240	5	30	240
Kidney	43	72	82	—	—	—
Liver	59	100	—	42	81	100
Lung	73	100	100	77	66	83
Striated muscle	45	100	100	—	86	100

The metabolism of histamine by the different organs was found to be a rapid process, as can be seen from Table 5. When the metabolic rate was measured per gram of tissue the lung appeared to have considerable metabolic activity compared with the other organs. However, no definite conclusions can be drawn from this observation because of the rapid turnover and metabolism of histamine by the different tissues, which enable histamine and its metabolites to be transported from one organ to another without definite proof that the histamine was catabolized in any particular organ. It is clear that the cellular destruction of histamine in the rat is a very rapid process. Exclusion of the kidneys from the circulation did not alter this rate of degradation of biologically active histamine by the tissues, as can be seen from Table 4.

The excretion of both forms of histamine in the urine was measured during a period of 4 hr after the injection of the histamine. After 30 min 27 %, and after 240 min 76 % of the injected histamine had been excreted in the form of radioactive ^{14}C (Fig. 3). The quantity of biologically active histamine varied between 15 and 45% of the injected histamine. From Tables 3 and 5 it can be seen that the destruction of the biologically active histamine was almost complete after 4 hr. It would therefore be expected that the renal excretion of the biologically active histamine and its metabolites would be almost complete at the end of this period also. The figures for the recovery of biologically active histamine and its metabolites in the urine confirm this, and are in close agreement with those obtained by Schayer (1952) in the rat. He recovered 95% of the injected radioactive histamine 20 hr after its injection, of which 11% was in the form of chemically unchanged histamine.

DISCUSSION

Between 90 and 95% of the radioactive histamine which was injected in the present experiments was accounted for in the blood and tissues. This confirms that the method for extracting and measuring the radioactivity was sufficiently quantitative to allow valid conclusions to be drawn from the experimental results. The normal content of biologically active histamine in the different tissues of the rats showed considerable variation, but a mean value for it was obtained in a preliminary experiment on twelve animals of the same strain, so that the value for the actual uptake by the tissues was obtained by subtracting the normal value from the value obtained after injection of the exogenous histamine. The high value for the skin histamine made it impossible to obtain accurate results for the uptake of exogenous biologically active histamine in this tissue.

The histamine diffused rapidly through the circulating blood after its intravenous injection. If the figures for the concentration of histamine in the plasma are used to calculate the volume of extracellular fluid in which the histamine was distributed 1 min after its injection, it was found to have a diffusion space of about 70 ml./100 g in the rat, which is greater than the total volume of water contained in the whole animal. Therefore the histamine must have diffused out of the extracellular fluid into the cells, where it was stored in concentrations higher than those found in the blood itself. Emmelin (1951) also concluded on the basis of his calculations that histamine injected intravenously into cats passed in large quantities to the tissues, where it was subsequently inactivated. These observations do not agree with Schayer's statement (1956) concerning the fate of exogenous histamine in the rat. In the present experiments on rats the radioactive histamine was absorbed by all tissues with the exception of the central nervous system. The quantity absorbed varied from one tissue to another; it was greatest for both radioactive and biologically active histamine in the kidneys. It is possible that exogenous histamine is deposited in the cells in a biochemical form different from that in which endogenous histamine is deposited.

Study of the distribution of histamine and its metabolites in the normal animal is made difficult by their rapid excretion through the kidneys. Exclusion of the kidneys from the circulation overcame this difficulty, and the two-stage character of the operation prevented undesirable effects produced by complete bilateral nephrectomy on the day of the experiment. In the nephrectomized animals the biologically active histamine in the blood decreased progressively while the radioactivity persisted at a constant level from 15 min until several hours after the injection. The biologically active histamine was being progressively metabolized, and a diffusion equilibrium between the histamine and its inactive metabolites became established across the cell membranes. During this period there was also a transfer of radioactive ^{14}C from liver and other abdominal viscera to striated muscle and lungs.

The present results show that the metabolism of histamine by the tissues is extremely rapid, almost 100 % of the histamine taken up by the tissues being metabolized in 30 min. It is clear that exogenous histamine in normal animals can be metabolized in large quantities and this metabolic process appears to occur especially in the kidney. It was found that the kidney had considerable power of extracting histamine from the blood and that a small proportion of this histamine was excreted in a chemically unchanged form.

SUMMARY

1. By the intravenous injection of radioactive ^{14}C histamine, the turnover, tissue distribution and fate of histamine has been investigated in the rat. The value of this method of investigation lay in the possibility of making comparisons between the concentrations for the radioactive ^{14}C and the biologically active histamine introduced into the body. The radioactive ^{14}C measured the total histamine concentration both in its biologically active form and after its metabolic transformation in the tissues.

2. Diffusion of the histamine out of the blood occurred very rapidly during the first few seconds after its injection. The factors which modified its rate of diffusion were investigated in normal rats, and in rats whose kidneys were excluded from the circulation.

3. Histamine penetrated rapidly into the cells of all the tissues except the brain. The extent of the uptake varied in the different organs. The kidney had the greatest power of extracting histamine from the blood.

4. Histamine taken up by the tissues was rapidly metabolized and the value for the fraction $\frac{\text{biologically active histamine}}{\text{radioactive } ^{14}C}$ correspondingly diminished.

5. The rate at which histamine was metabolized in the extracellular fluid and in the different organs was determined. In the rats whose kidneys were excluded from the circulation, the metabolic products of histamine were gradually transferred between the tissues.

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MEMBRANE CONSTANTS OF MAMMALIAN MUSCLE FIBRES

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Preliminary estimates of the resistance and capacitance of the mammalian muscle fibre membrane have been reported previously (Boyd & Martin, 1956*b*). In the present paper the results of experiments in which the membrane constants were measured will be presented in greater detail. The constants were determined by the method of 'square pulse analysis' (Hodgkin & Rushton, 1946; Katz, 1948; Weidmann, 1951, 1952; Fatt & Katz, 1951), intracellular electrodes being used to pass current through the membrane and to record the resultant potential changes.

METHODS

The isolated tenuissimus muscle of the cat was used in all experiments and was mounted in a constant-temperature bath containing oxygenated Krebs's solution. The experimental methods have been described in detail in a previous paper (Boyd & Martin, 1956*a*). The electrical arrangement for measuring current and potential across the fibre membrane with two internal electrodes was similar to that described by Fatt & Katz (1951, p. 325, fig. 4).

The two micro-electrodes were inserted into the fibre as close together as possible, and a rectangular current pulse passed through one. The resultant change in membrane potential was recorded by the other (e.g. Fig. 1*a*). The recording electrode was then moved to between 0·5 and 1·0 mm from the current electrode and the procedure repeated (Fig. 1*c*). Finally the recording electrode was returned to an intermediate position and a third set of records obtained (Fig. 1*b*).

Resting potentials ranged from 55 to 70 mV when the records were obtained, generally dropping to about 5 mV below their initial value on insertion of the second micro-electrode, and thereafter decaying slowly. If the resting potential had fallen to below 55 mV when the last record was obtained or if, when the results were plotted as in Fig. 2, the intermediate point did not fall reasonably close to the straight line joining the first two, the measurements were rejected.

RESULTS

In Fig. 1 records are shown of current pulses and electrotonic potentials obtained from one fibre at three different electrode separations. The electrotonic potentials rise in less than 10 msec to a steady value determined for a given

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current by the distance between the electrodes. Applying the cable theory of Hodgkin & Rushton (1946), the potential change, V, produced by a steady current, I, through the membrane is given by the equation

$$V = \tfrac{1}{2} I \sqrt{(r_m r_i)} \exp\left[-x/\sqrt{(r_m/r_i)}\right],$$

where x is the electrode separation, r_m the transverse resistance of a unit length of fibre membrane and r_i the internal longitudinal resistance per unit length of fibre. The term $\sqrt{(r_m/r_i)}$ is the space constant, λ, of the fibre.

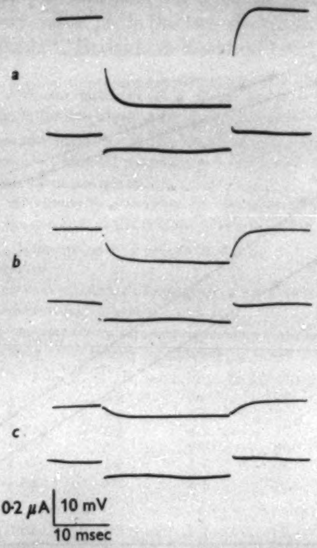

Fig. 1. Electrotonic potentials produced by current pulses with three different electrode separations. Lower record in each case is current pulse. Interelectrode distances were: *a*, 0·05 mm; *b*, 0·60 mm; *c*, 1·10 mm. mV scale applies to electrotonic potentials, μA scale to current pulses. Normal Krebs's solution; 24° C.

The results of four experiments, obtained from records similar to those illustrated in Fig. 1, are shown in Fig. 2. The ratio $V:I$ is plotted on a logarithmic scale against the separation of the micro-electrodes. In each case the experimental points fall on a straight line, as predicted by the theory. λ may be obtained from the slope of the line (being the distance from any point on the fibre over which the electrotonic potential falls to 1/e of its value at that point), and $\tfrac{1}{2}\sqrt{(r_m r_i)}$ from the point at which the line intersects the vertical axis ($x = 0$). From these two values r_m and r_i can be calculated.

Further calculations require an estimate of the resistivity of the myoplasm

(R_i). This was estimated from the value of 250 Ω cm reported for frog myoplasm (Bozler & Cole, 1935; Katz, 1948), allowing for the higher ionic concentrations in the mammalian muscle and applying a temperature correction. The conductivity ($1/R_i$) was assumed to be proportional to the ionic concentration and to increase with temperature with a Q_{10} of 1·3 (Hartree & Hill, 1921).

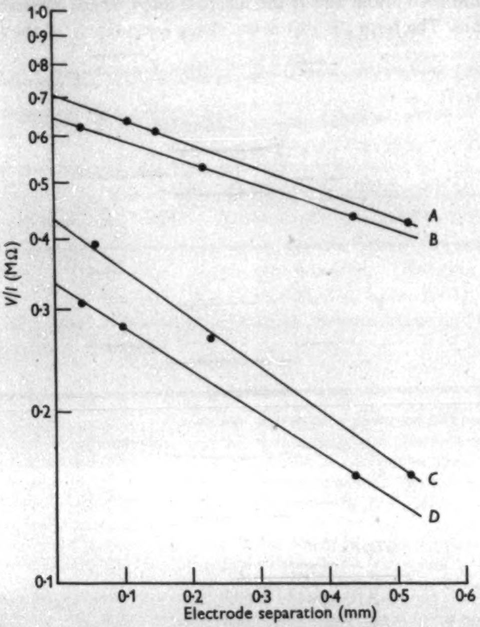

Fig. 2. Spatial decay of electrotonic potentials in four fibres. Ordinate, amplitude of electrotonic potential, when a steady state is reached, divided by amplitude of current pulse; logarithmic scale. Abscissa, separation between current and voltage recording electrodes. Fibres *A* and *B*, 37° C; fibres *C* and *D*, 22° C.

These assumptions result in an estimate of 125 Ω cm for the specific resistance of mammalian myoplasm at 37° C, which is close to the mean value of 105 Ω cm (range 52–190) reported by Weidmann (1952) for the internal resistivity of kid Purkinje fibres. Values used for R_i at other temperatures are based on the same assumptions.

Knowing R_i, the fibre radius, ρ, may be obtained from the relation

$$\rho = \sqrt{(R_i/\pi r_i)}.$$

The transverse resistance of a unit area of membrane is then given by

$$R_m = 2\pi\rho r_m$$

and the membrane capacitance per unit area by

$$C_m = \tau_m / R_m.$$

τ_m, the time constant of the membrane, may be obtained from the rising or falling phase of the electrotonic potential. Two methods were used:

(*a*) τ_m in Tables 1 and 2—the time taken for the potential to rise to 83% of its maximum steady value, with the two electrodes as close together as possible ($x/\lambda = 0{\cdot}1$, Table 1, Hodgkin & Rushton, 1946).

TABLE 1. Data derived from one muscle in which curves were obtained, similar to those in Fig. 2, for five fibres at 37° C and five at 22° C

r_m, transverse resistance of unit length of membrane.
r_i, internal longitudinal resistance per unit length of fibre.
R_i, resistivity of myoplasm.
τ_m, τ_m', membrane time constant by two methods (see text).
λ, fibre space constant (from slope of line as in Fig. 2).
$\frac{1}{2}\sqrt{(r_m r_i)}$, intercept on $V:I$ axis as in Fig. 2.
ρ, fibre radius.
R_m, transverse resistance of unit area of membrane.
C_m, membrane capacitance per unit area.

Fibre	$\frac{1}{2}\sqrt{(r_m r_i)}$ (Ω)	$\lambda = \sqrt{(r_m/r_i)}$ (mm)	τ_m (msec)	τ_m' (msec)	ρ (μ)	R_m (Ω cm²)	C_m (μF/cm²)
			A. 37° C (R_i assumed to be 125 Ω cm)				
I	$6{\cdot}4 \times 10^5$	1·10	5·6	4·1	19	1620	3·5
II	$3{\cdot}7 \times 10^5$	1·10	4·7	4·4	25	1230	3·8
III	$7{\cdot}0 \times 10^5$	1·00	4·5	4·1	17	1490	3·0
IV	$3{\cdot}8 \times 10^5$	1·30	5·0	3·5	26	1600	3·1
V	$4{\cdot}8 \times 10^5$	1·00	4·8	5·7	21	1220	3·9
Mean		1·10	4·9	4·4	22	1430	3·5
			B. 22° C (R_i assumed to be 195 Ω cm)				
I	$3{\cdot}4 \times 10^5$	0·60	1·2	1·3	23	570	2·1
II	$2{\cdot}8 \times 10^5$	0·60	1·7	1·4	26	555	3·1
III	$3{\cdot}7 \times 10^5$	0·75	2·1	2·6	25	872	2·4
IV	$4{\cdot}9 \times 10^5$	0·58	1·6	3·0	19	688	2·3
V	$4{\cdot}5 \times 10^5$	0·50	1·8	2·4	18	506	3·6
Mean		0·61	1·7	2·1	22	638	2·7

(*b*) τ_m' in Table 1—from the velocity of propagation of the half-value of the electrotonic potential following the make of the current pulse (Hodgkin & Rushton, 1946).

The capacitance values given in the tables were obtained using the values of τ_m from method (*a*).

The results of these calculations for ten fibres in one muscle are given in Table 1. Five sets of measurements were made at 37° C and the remainder at 22° C. The mean transverse membrane resistance was 1430 Ω cm² and the capacitance 3·5 μF/cm². At 22° C the membrane resistance was reduced to about 640 $\Omega\,cm^2$. In addition to this marked decrease in membrane resistance with temperature there was a decrease in the membrane capacitance to a mean of 2·7 $\mu F/cm^2$. The calculated values for the fibre diameters agree with those obtained by direct measurement from histological transverse sections of a tenuissimus muscle. Fibre diameters estimated from photographs ranged from 30 to 60μ.

The results obtained from the muscle of Table 1 and three other muscles are summarized in Table 2. In two of the muscles measurements were made only with electrode separation almost zero, and it was necessary to assume a value for the fibre diameter (50μ) in order to obtain values of R_m. However, the results are in good agreement with those obtained from the other two muscles.

TABLE 2. Comparison of the mean values of fibre constants at two different temperatures for four muscles. For muscle *A* and *B* fibre radius was calculated from the data; for *C* and *D* fibre radius was assumed to be 25μ. Symbols as in Table 1

Muscle	No. of fibres	Temp. (° C)	ρ (μ)	λ (mm)	τm (msec)	C_m ($\mu F/cm^2$)	R_m ($\Omega\,cm^2$)	Q_{10} of R_m
A	5	37	22	1·1	4·9	3·5	1430	1·71
	5	22	22	0·6	1·7	2·7	638	
B	4	37	18	1·0	4·1	2·8	1475	1·48
	4	22	21	0·7	1·7	2·1	822	
C	4	37	(25)	1·2	4·4	3·1	1400	1·62
	4	24	(25)	0·7	2·6	3·5	750	
D	10	37	(25)	1·2	5·6	4·0	1400	

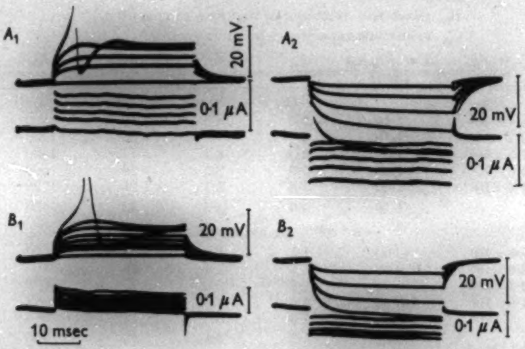

Fig. 3. Variation of membrane potential with applied current in two fibres, *A* at 37° C and *B* at 24° C. A_1, B_1, depolarization; A_2, B_2, hyperpolarization. Zero electrode separation. As depolarizing current pulse is increased a transient 'local response' and then an action potential appears on the electrotonic potential.

Since the theory on which the above analysis is based does not apply to a non-linear system, it is necessary to confirm the implicit assumption that the transverse resistance is independent of membrane potential. To do this two micro-electrodes were inserted into a muscle fibre as close to one another as possible, and the current and electrotonic potential recorded at various levels of polarization. The records from two fibres, one at 37° C and the other at 24° C, are shown in Fig. 3. The depolarizing current pulses were increased until a transient 'local response' and then an action potential appeared.

Measurements obtained from the records of Fig. 3 are presented graphically in Fig. 4. The amplitude of the electrotonic potentials when a steady state is reached is plotted against the magnitude of the current pulse. The voltage–current relation is linear from about 30 mV hyperpolarization to near threshold depolarization at both temperatures. Similar linear relationships were obtained for six other fibres.

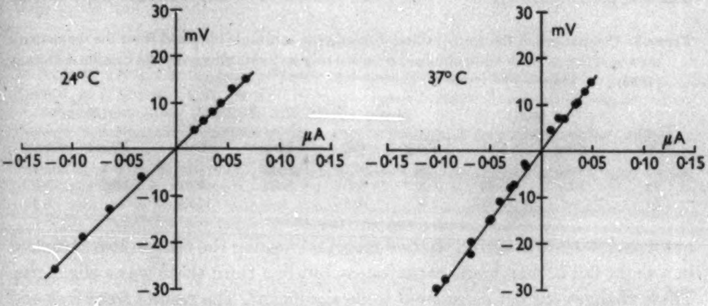

Fig. 4. Voltage-current relation in two fibres, one at 24° C and one at 37° C, plotted from records similar to those shown in Fig. 3. The voltage values represent the amplitude of the electrotonic potential when a steady state is reached, and depolarization is plotted upwards. The relation is linear from about 30 mV hyperpolarization to near threshold depolarization.

DISCUSSION

The results show that the transverse resistance of unit area of mammalian muscle fibre membrane at body temperature is about 1400 Ωcm^2, and the membrane capacitance per unit area between 2 and 4 μF/cm^2. The mean time constant for four muscles, of 4·8 msec at 37° C, is more than twice as large as that obtained from the rate of decay of the end-plate potential (about 2 msec; Boyd & Martin, 1956*b*), although in the present experiments the values obtained by two different methods of calculations are similar (Table 1). The value given for the membrane capacitance is, therefore, only an approximate one.

The calculated mean diameter of the muscle fibres is in good agreement with that found both in the one muscle sectioned in these experiments, and in a series of muscles subsequently examined (Boyd, 1956). In the latter the mean diameter of the surface fibres in fixed muscles was found to be 35μ; corrected for shrinkage this gives a diameter of about 50μ for fibres accessible to a micro-electrode. This was the value used in the calculations of the constants in muscles *C* and *D* of Table 2. In the muscles in which the mean fibre diameter was calculated, the good agreement between the values obtained at the two temperatures employed (e.g. Table 1) also suggests that the method used was satisfactory.

Lowering the temperature of the preparation by 15° C results in a fall in the transverse resistance to nearly half its value at body temperature (mean $Q_{10} = 1{\cdot}6$). This is in contrast to the effect of change in temperature on frog muscle; del Castillo & Machne (1953) obtained a negative temperature coefficient of about 1·35 for the membrane resistance of frog sartorius fibres. The temperature range over which the two series of experiments were carried out was different, however. In two muscles lowering the temperature resulted in a slight fall in membrane capacitance, but in a third there was a slight rise. These changes are not considered to be significant. The results from frog and cat are compared in Table 3, and it will be seen that the values of all the constants are much smaller for the mammalian membrane than for that of the frog, in addition to the fact that the temperature coefficients over the ranges shown are of opposite sign.

TABLE 3. Comparison of the mean values of membrane constants obtained from the tenuissimus muscle of the cat with those obtained from the frog sartorius muscle by del Castillo & Machne (1953)

Temp. (°C)	λ (mm)		τ_m (msec)		R_m (Ω cm²)		C_m (μF/cm²)	
	Frog	Cat	Frog	Cat	Frog	Cat	Frog	Cat
3	1·82	—	34	—	3643	—	9·7	—
22	1·59	0·67	22	2·0	2065	730	10·6	2·8
37	—	1·05	—	4·8	—	1453	—	3·4

Del Castillo & Machne (1953) found that the influence of temperature on the transverse resistance of the frog muscle membrane does not differ significantly from that on the conductivity of a salt solution of about the same ionic concentration as that normally surrounding the muscle fibres. The decrease in membrane resistance in mammalian muscle when the temperature is reduced, however, probably indicates an increase in the permeability of the membrane to one or more of the ions present. It is interesting to note that the values of the resting potential of the mammalian fibre membrane were found to be consistently lower (by about 5–10 mV) when the preparation was at room temperature than when it was maintained at body temperature. No similar change has been reported for frog muscle.

SUMMARY

1. The membrane constants of mammalian skeletal muscle fibres have been measured by 'square pulse analysis'.

2. The transverse resistance of the fibre membrane at 37° C is about 1400 Ω cm^2, and the membrane capacitance lies between 2 and 4 μF/cm^2.

3. The transverse resistance falls with a Q_{10} of 1·6 when the temperature is lowered from 37 to 22° C, while the membrane capacitance is not altered significantly.

4. The results were compared with those obtained by del Castillo & Machne (1953) for frog muscle and the marked differences discussed.

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PLASMA-KININ-FORMING ENZYMES IN BODY FLUIDS AND TISSUES

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It has been proposed that all vasodilator and smooth-muscle-stimulating polypeptides derived from plasma proteins should be called plasma kinins, until it has been found whether they all possess the same structure or whether they are a group of related polypeptides. The enzymes which form these plasma kinins, designated plasma-kinin-forming enzymes, are the subject of the present investigation. It has been found that there are at least two types of enzymes which form plasma kinins when incubated with plasma globulins. One type acts quickly and is not inhibited by proteolytic inhibitors, the other acts slowly, is inhibited by proteolytic inhibitors and may act through activation of plasminogen to plasmin.

METHODS

The experiments were carried out with skin extracts, saliva, lachrymal secretion, pancreatic juice, urine and urokinase. In order to test whether the skin extracts and body fluids acted upon plasma proteins to form a smooth-muscle-stimulating substance, samples were incubated with pseudoglobulin solution at 34° C and the incubated mixture was tested on the isolated guinea-pig ileum suspended in a 15 ml. organ bath containing Mg-free Tyrode solution at 34° C. In experiments in which the plasma-kinin-forming activity was determined at various times of incubation of a given mixture, the experiment was not considered valid unless the sensitivity of the tissue to a standard plasma kinin solution remained constant throughout.

Preparation of an acetone-dried skin. Samples of rat skin from the abdomen and of cat skin from the thigh were used. The skin areas to be extracted were shaved. The rats were killed by a blow on the head, exsanguinated, and the skin removed at once. In cats the skin was removed under pentobarbitone anaesthesia. The skin samples were immediately frozen in solid CO_2, weighed while still frozen, placed in acetone and chopped with scissors in acetone. In some experiments 20 mμ sections of the frozen skin were cut and placed in acetone as described by Beloff & Peters (1945), but no more activity was extracted than from the chopped skin. The chopped skin was washed with 25 ml. acetone per gram of skin three or four times, and the sediment was separated each time by centrifugation. This procedure gave an acetone-dried preparation which was about one quarter of the original skin weight (1–5 g.).

Extraction of a plasma-kinin-forming enzyme from skin. Saline or Tyrode extracts of the acetone-dried skin contain no plasma-kinin-forming enzyme, because the proteolytic enzymes of the skin remain firmly bound, making them insoluble. Therefore some procedure had to be adopted in order to free the enzymes from the structural elements. Beloff & Peters (1945) used strong solutions of potassium chloride to 'salt out' their proteolytic enzyme. This procedure did not facilitate the extraction of the plasma-kinin-forming enzyme, nor did a solution of potassium phosphate (2 M at pH 7·5). On the other hand, a 2 M potassium thiocyanate solution used by Astrup & Permin (1947) to extract plasminogen activator from hog heart muscle proved effective. The thiocyanate probably serves to disintegrate the tissue, allowing the enzyme to be separated from the main cell structures.

The procedure adopted in the present investigation is a modification of the method used by Astrup & Albrechtsen (1957). The acetone-dried skin was ground with washed sand in 2 M potassium thiocyanate, 10 ml./g skin (wet weight), allowed to stand for 30 min and then centrifuged (3000 rev/min) for 20 min at 5° C. This was repeated three times. The potassium thiocyanate solutions were combined, diluted seven times with distilled water, and made acid to pH 2 by the addition of 0·1 N-HCl. A fine precipitate formed and the suspension was allowed to stand for 1 hr at 5° C, and then centrifuged at 5° C for 20 min (3000 rev/min).

In the method described by Astrup & Albrechtsen (1957) the precipitate formed after acidification of the thiocyanate was taken up and dissolved in fresh thiocyanate, and this solution was used for the estimation of plasminogen activator. In the present experiments the plasma-kinin-forming activity could not be estimated in potassium thiocyanate solution. Therefore the precipitate obtained on acidifying the thiocyanate extract was suspended in Tyrode solution, 1 ml. containing extract from 100 mg of skin (wet weight). In this suspension, however, the enzyme activity remained associated with the solid phase and was not in solution.

Saliva. Human saliva was collected after washing the mouth with saline solution. Cat and dog saliva was collected from a cannula in Wharton's duct under pentobarbitone anaesthesia and secretion was stimulated by intramuscular injection of pilocarpine 1 mg/kg. The saliva so collected was sometimes used after dilution with saline; at other times it was either freeze-dried and stored at −10° C or precipitated with 95% acetone and kept at −10° C. The activity was qualitatively similar in all three procedures. The acetone-dried saliva usually contained 70–80% of the activity of the original saliva.

Lachrymal secretion was collected directly from the corneal surface of cats under pentobarbitone anaesthesia, after injection of pilocarpine 1 mg/kg.

Pancreatic juice. Samples of pancreatic juice were obtained from cannulated pancreatic ducts of anaesthetized cats. Two types of secretion were examined. Secretin juice secreted after intravenous injection of secretin, and pancreozymin juice secreted after intravenous injection of pancreozymin (Harper & Raper, 1943).

Urine. Human urine dialysed against running tap water for at least 4 hr was used. In some experiments urokinase was used. Urokinase is the name given by Ploug & Kjeldgaard (1956, 1957) to an enzyme prepared from human urine which activates plasminogen.

Human plasminogen. Human plasminogen was prepared by the method of Kline (1953); the final solution was freeze-dried and stored at −10° C. Citrated human plasma from the Edgware Transfusion Centre was used in its preparation.

Estimation of plasminogen activation. Rat skin extract, undiluted human saliva, acetone-dried saliva and urokinase were tested for the presence of a plasminogen activator. Samples were incubated with plasminogen and casein and proteolysis was determined by the hydrolysis of casein, measuring the increase in optical density at 275 mμ of the deproteinized reaction mixture, as described by Mullertz (1955).

Pseudoglobulin solution was prepared from dog plasma and consisted of the fraction of plasma protein precipitated by $(NH_4)_2SO_4$ 33–46% saturation. This precipitate was dialysed for 48 hr against running tap water and the final solution (one third of the original volume) freeze-dried in 5 ml. ampoules and stored at −10° C.

RESULTS

The skin extracts as well as all the body fluids tested, such as saliva, lachrymal secretion, pancreatic juice, and urine, formed plasma kinins when incubated with pseudoglobulin (for references see Hilton & Lewis, 1957). The time course of plasma kinin formation, however, differed with the skin extracts and the different body fluids. Further, the enzymes present in these different materials showed differences in their susceptibility to such proteolytic inhibitors as soya-bean trypsin inhibitor and antiplasmin, suggesting the presence of two plasma-kinin-forming enzymes.

Skin extracts. The thiocyanate extracts did not cause contraction of the guinea-pig ileum, nor did they affect the contraction caused by a standard preparation of a plasma kinin. When the extracts, however, were incubated with pseudoglobulin solution, smooth-muscle-stimulating activity appeared in the incubated mixture. This result was not obtained with all skin extracts. Some were also inactive after incubation with pseudoglobulin. Figure 1 illustrates an experiment with an active skin extract. The smooth-muscle-stimulating activity at *c* and *e* was obtained after 20 min incubation of pseudoglobulin with a thiocyanate extract of rat's skin. Incubation of the pseudoglobulin with Tyrode solution did not lead to the appearance of smooth-muscle stimulating activity as shown at *d*.

Fig. 1. Guinea-pig ileum suspended in 15 ml. Tyrode solution. At (*a*) 1 ml. rat skin extract; at (*b*) plasma kinin 10 μg; organ bath not washed out between (*a*) and (*b*). At (*c*) 1 ml. and at (*e*) 0·5 ml. rat skin extract incubated for 20 min with pseudoglobulin solution. At (*d*) same amount pseudoglobulin solution as at (*c*) incubated for 20 min with Tyrode solution.

As the activity of the thiocyanate extracts was associated with the solid phase of the suspension, it was not possible to estimate quantitatively the amount of plasma-kinin-forming enzyme in skin. Further, some of the extracts contained an inhibitor of the plasma-kinin-forming enzyme. This was evident when an active extract which formed plasma kinin on incubation with pseudoglobulin was incubated with an inactive extract in addition to the pseudoglobulin. No plasma kinin formation occurred. Thus a low yield of activity on incubation of an extract with pseudoglobulin need not be a sign of a low content of plasma-kinin-forming enzyme in the extract, but could result from the combined presence of large amounts of enzyme with an inhibitor.

The plasma kinin content of a mixture of active skin extract and pseudoglobulin increased with the time of incubation, reaching a maximum after about 20 min. After incubation for 20 min the plasma kinin content usually decreased owing to the presence of a plasma-kinin-destroying enzyme in the pseudoglobulin solution. In the experiment of Fig. 2 an active skin extract was tested at various intervals of time during a 50 min period of incubation with pseudoglobulin. Formation and destruction of plasma kinins were apparently going on together at the same rate between the 20th and 50th minute, since during this period the amounts of kinin in the mixture remained the same. The plasma kinin formation by skin extracts was inhibited by the soya-bean trypsin inhibitor and by antiplasmin. This is illustrated in Fig. 3.

Fig. 2. Guinea-pig ileum suspended in 15 ml. Tyrode solution. Rat skin extract (0·5 ml.) and pseudoglobulin after incubation for 90 sec, at (*a*), 5 min at (*b*), 10 min at (*c*), 15 min at (*d*), 20 min at (*e*), 25 min at (*f*), 30 min at (*g*), 50 min at (*h*).

Saliva. The plasma-kinin-forming enzyme in saliva behaves differently from the enzyme in skin extracts. On incubation of saliva with pseudoglobulin the maximum formation of plasma kinin occurs within 5 min and sometimes in less than 3 min. In the experiment of Fig. 4 human saliva, diluted 1:100, was incubated for various intervals of time with pseudoglobulin. Maximum formation of plasma kinin occurred between 90 sec and 5 min, and after 20 min incubation the mixture contained hardly any plasma kinin. This result is in contrast to that of the experiment with skin extract illustrated in Fig. 2, although in both experiments pseudoglobulin from the same batch and in the same concentration was used. The difference in the rate of formation of plasma kinin in the two experiments is not due to the presence, either in saliva or in skin extract, of some factor interfering with the formation or destruction of the plasma kinin, but to the fact that the plasma-kinin-forming enzyme in skin extract is different from that in saliva. This is evident when saliva and skin extract are incubated together with pseudoglobulin. Summation of quick and slow plasma kinin formation occurs. The result of such a summation experiment is illustrated in Fig. 5. The two enzymes thus act independently; one acts quickly and the other acts slowly. The plasma-kinin-forming enzyme of saliva, unlike that of skin extract, was not inhibited by either soya-bean trypsin inhibitor or antiplasmin.

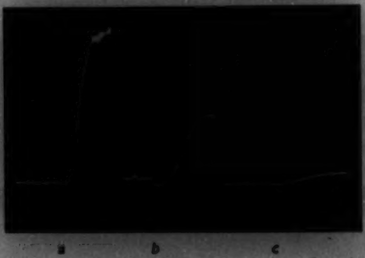

Fig. 3. Guinea-pig ileum suspended in 15 ml. Tyrode solution. Rat skin extract (1 ml.) after incubation for 20 min with pseudoglobulin at (*a*), with pseudoglobulin and soya-bean trypsin inhibitor (100 μg/ml.) at (*b*) and with pseudoglobulin and antiplasmin (3 mg/ml.) at (*c*).

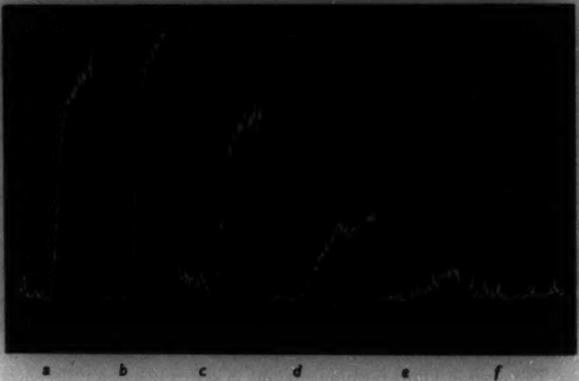

Fig. 4. Guinea-pig ileum suspended in 15 ml. Tyrode solution. Human saliva (0·2 ml. of 1:100) after incubation with pseudoglobulin for 90 sec at (*a*), 5 min at (*b*), 10 min at (*c*), 15 min at (*d*), 20 min at (*e*) and 30 min at (*f*).

Lachrymal secretion. The plasma-kinin-forming enzyme in lachrymal secretion appears to be a quick-acting enzyme resembling that in saliva. On incubation of lachrymal secretion with pseudoglobulin, plasma kinins were found in the mixture after 90 sec and 5 min, but no longer after incubation for 15 min.

Pancreatic juice. The results obtained with secretin juice and pancreozymin juice were different. With secretin juice the formation of plasma kinin, on incubation with pseudoglobulin, followed the same time course as in the experiments with saliva. When pancreozymin juice was incubated with pseudoglobulin, formation of plasma kinin also commenced within 90 sec and decreased within 5 min but later there followed a renewed period of plasma kinin formation which continued for more than 30 min, suggesting that pancreozymin juice contained both types of enzymes, the quick-acting enzyme of saliva and the slow-acting enzyme of skin extracts. These results are illustrated in Fig. 6. The quick-acting enzyme in secretin juice and in pancreo zymin juice was not affected when incubation with pseudoglobulin was carried out in the presence of either soya-bean inhibitor (1 mg/ml.) or antiplasmin (3 mg/ml.). However, the slow-acting enzyme in the pancreozymin juice was partly inhibited under these conditions.

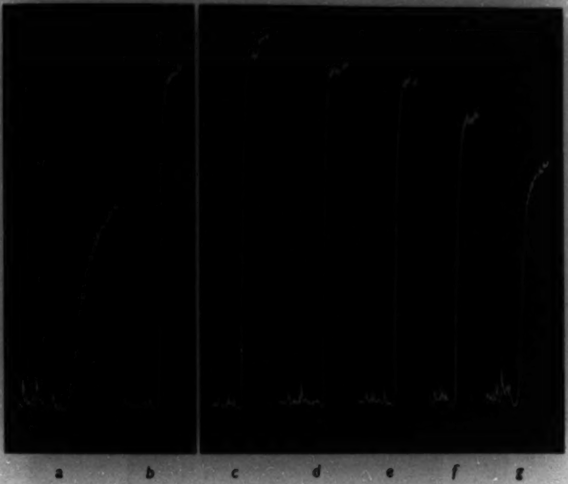

Fig. 5. Guinea-pig ileum suspended in 15 ml. Tyrode solution. Rat skin extract (0·5 ml.) and human saliva (0·2 ml. of 1:100) after incubation with pseudoglobulin for 90 sec at (*a*), 5 min at (*b*), 15 min at (*c*), 20 min at (*d*), 25 min at (*e*), 35 min at (*f*), and 50 min at (*g*).

Urine. The formation of plasma kinins by urine also results from the activity of two enzymes. This is evident when the plasma kinin formation is studied in the presence or absence of the soya bean inhibitor. Such an experiment is illustrated in Fig. 7. On incubation of dialysed human urine with pseudoglobulin alone (*A*) most of the plasma kinins formed appeared to be produced by the quick-acting enzyme. But in the presence of the soya-bean inhibitor (*B*) there was some reduction of plasma kinin formation in the samples tested after incubation for 15, 25, 35 and 45 min, suggesting that the slow-acting enzyme which is sensitive to this proteolytic inhibitor is present in the urine as well.

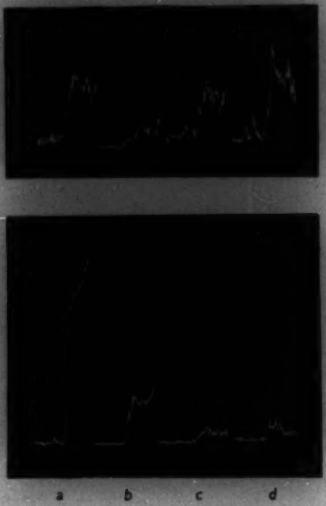

Fig. 6. Guinea-pig ileum suspended in 15 ml. Tyrode solution. Pancreatic juice after incubation with pseudoglobulin for 90 sec at (*a*), 5 min at (*b*), 15 min at (*c*) and 30 min at (*d*). Upper record, 'pancreozymin' juice; lower record, 'secretin' juice.

The existence of a slow-acting plasma-kinin-forming enzyme in urine is confirmed by the fact that urokinase prepared from human urine is a slow-acting plasma-kinin-forming enzyme which is sensitive to proteolytic inhibitors. The time course of plasma kinin formation by urokinase and its inhibition by antiplasmin is illustrated in the experiment of Fig. 8.

Activation of plasminogen. The finding that urokinase activates plasminogen to plasmin was confirmed. In these experiments casein was used as substrate and was incubated with urokinase (50–100 μg/ml.) and human plasminogen. The casein was not hydrolysed when incubated with urokinase alone.

No plasminogen activator was found in saliva, since no hydrolysis occurred when the casein was incubated with human plasminogen and saliva (1–20 μg/ml.). For these experiments undiluted saliva as well as acetone-dried saliva was used. The failure of saliva to activate plasminogen could not be attributed to the presence of an inhibitor, since saliva was found not to inhibit the activity of urokinase. No plasminogen activator was detected in skin extract when it was suspended in Tyrode solution and incubated with human plasminogen and casein.

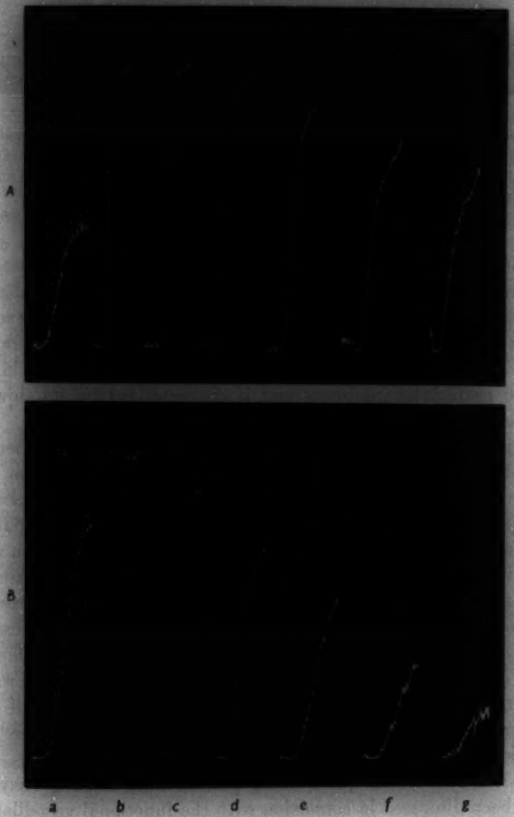

Fig. 7. Guinea-pig ileum suspended in 15 ml. Tyrode solution. Dialysed human urine (0·3 ml.) after incubation with pseudoglobulin alone (*A*) and after incubation with pseudoglobulin and 0·5 mg/ml. soya bean trypsin inhibitor (*B*). Mixtures after incubation for 45 sec at (*a*) 4·5 min at (*b*), 9 min at (*c*), 15 min at (*d*), 25 min at (*e*), 35 min at (*f*) and 45 min at (*g*).

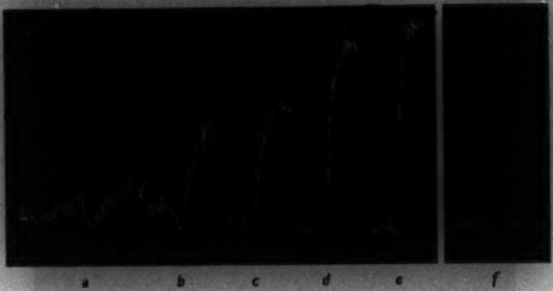

Fig. 8. Guinea-pig ileum suspended in 15 ml. Tyrode solution. Urokinase (250 μg) after incubation with pseudoglobulin for 2 min at (*a*), 6 min at (*b*), 11 min at (*c*), 18 min at (*d*), 24 min at (*e*). At (*f*) urokinase (250 μg) after incubation for 23 min with pseudoglobulin and 3 mg/ml. antiplasmin.

DISCUSSION

The present experiments provide evidence that there are two enzymes which form plasma kinin when body fluids or tissue extracts are incubated with plasma globulins. The one enzyme, present in urine and in glandular secretions, is resistant to proteolytic inhibitors and is characterized by the rapidity of its action. The second enzyme, present in skin extracts and also in urine, is sensitive to proteolytic inhibitors and is characterized by its slow rate of formation of plasma kinin.

As plasmin is also a slow-acting plasma-kinin-forming enzyme, sensitive to the proteolytic inhibitor antiplasmin (Lewis, 1958) the question arises of whether the slow-acting enzymes in skin extract and in urine form plasma kinin by first activating plasminogen in the pseudoglobulin preparation to plasmin. In favour of this idea is the finding that the plasminogen activator urokinase, which is derived from urine, acts like the slow-acting plasma-kinin-forming enzyme and is inhibited by antiplasmin and soya-bean trypsin inhibitor. On the other hand, no evidence could be obtained for the presence of a plasminogen activator in skin extract. For these experiments, however, a suspension of a crude extract was used and plasma kinin formation was tested by a method which might have been too insensitive if only small amounts of activator had been present. Their detection might have required a more sensitive biological test for plasma kinin formation.

The possible role of the quick-acting plasma-kinin-forming enzymes has previously been discussed in connexion with the functional vasodilatation in the salivary glands and tongue (Hilton & Lewis, 1957) and in the sweat glands (Fox & Hilton, 1958). It was concluded that the plasma-kinin-forming enzyme escapes from the gland cells during activity into the interstitial fluid and there forms plasma kinin which causes the accompanying vasodilatations. The finding that other glandular secretions contain quick-acting plasma-kinin-forming enzymes, e.g. pancreatic and lachrymal secretions, suggests that this mechanism may occur in all glands.

The function of the slow-acting enzyme or activator of plasminogen is not clear. Such an enzyme in the skin might fulfil a role in local tissue reactions to injury as has been previously suggested by many workers. In addition, it might account for the vasodilatation produced by antidromic stimulation of sensory nerves, but its presence in urine and in certain pancreatic juices suggests further functions.

It cannot be assumed that the plasma kinins formed by the slow-acting enzymes are the same as those formed by the quick-acting enzymes. They are indistinguishable when examined on smooth-muscle preparations, but neither has been purified sufficiently to examine its actions in detail or to attempt a determination of its structure. The possibility therefore remains that the polypeptides formed by the quick- and the slow-acting plasma-kinin-forming enzymes have different chemical structures and different pharmacological actions.

SUMMARY

1. A plasma-kinin-forming enzyme has been extracted from the skin of rats and cats. It is a slow-acting enzyme which is inhibited by antiplasmin and soya-bean trypsin inhibitor.

2. The skin enzyme behaves like plasmin and like the plasminogen activator urokinase, which is extracted from human urine.

3. The possibility is discussed that the skin enzyme forms plasma kinin indirectly by first activating plasminogen to plasmin.

4. Another plasma-kinin-forming enzyme is present in the secretions of the salivary, lachrymal and pancreatic glands as well as in urine. It is a quick-acting enzyme which is not affected by antiplasmin or soya-bean trypsin inhibitor.

5. It is suggested that the slow-acting enzyme in skin plays a part in the local inflammatory reaction to injury, whereas the plasma kinins formed by the quick-acting enzyme are responsible for functional vasodilatation in glandular tissue.

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THE TRANSPORT OF RADIOACTIVE CARBON DIOXIDE IN THE BLOOD STREAM OF THE DOG AFTER ADMINISTRATION OF RADIOACTIVE BICARBONATE

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An understanding of the processes taking part in the transport of radioactive carbon dioxide and bicarbonate in the animal body is of central importance in the interpretation of experiments concerned with the oxidation *in vivo* of carbon-labelled compounds. In this connexion a number of papers have appeared which deal with the rate of elimination in the expired air of radioactive material injected in the form of bicarbonate; these include the experimental studies of Kornberg, Davies & Wood (1952) and of Drury, Wick & Almen (1956), together with more theoretical contributions such as that of Steele (1955). In the present communication results are presented on the transfer of radioactive carbon dioxide into and out of a particular region of the body, in this case the hind limb, following the intravenous injection of $NaH^{14}CO_3$. The results were collected as a preliminary to other experiments to be separately reported in which the behaviour of radioactive carbon dioxide in the same and other regions of the body was examined after the administration of glucose and other metabolites labelled with ^{14}C. Some of these results have already been briefly reported (Coxon & Robinson, 1956; Robinson & Coxon, 1957).

METHODS

Animals. The experiments were performed upon mongrel dogs weighing between 10 and 30 kg, kept without food for 24 hr before the start of the experiment.

Operative procedure. The dog was anaesthetized by an intraperitoneal injection of pentobarbital sodium, and a tracheal cannula was introduced for collection of expired air. This was done to avoid atmospheric contamination, whether or not the air was to be analysed in a particular instance. One femoral artery was ligated and cannulated for collection of arterial samples, and one or both external jugular veins were generally exposed, the left being generally employed for injection of the radioactive material and the right for cardiac catheterization if this was to be carried out. Hind-limb venous blood was collected from the femoral vein contralateral to the ligated artery by means of a cannula (Guest, 1941) which could be left in position without appreciably obstructing blood flow in the vessel. Mixed venous blood was collected through a catheter introduced into either the right side of the heart or the pulmonary artery via the right external jugular vein. The placing of the catheter was carried out under fluoroscopic control while the animal lay in a plastic tray which was transparent to X-rays. The general arrangement of the apparatus is shown in Fig. 1. The cardiac catheter, when used, and also the femoral venous needle were kept patent by the continuous infusion of a 0·9% solution of NaCl containing heparin 1000 i.u./100 ml. The rate of infusion was about 0·5 ml./min and this was roughly equivalent to the rate of blood loss from sampling in most instances.

Injection of radioactive material. Sodium ^{14}C bicarbonate was obtained from the Radiochemical Centre, Amersham. The dose used was between 5 and 20 μc contained in 10^{-3} m.-mole of salt solution; it was made up in 5 ml. of 0·9% NaCl solution and injected rapidly into the external jugular vein.

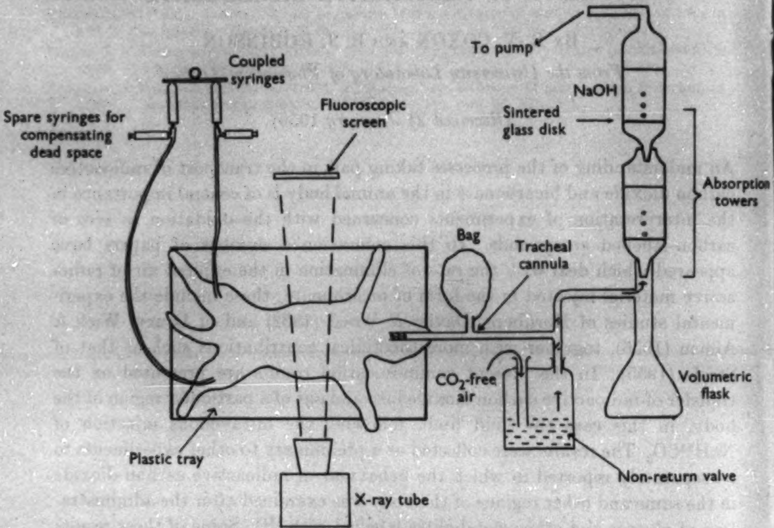

Fig. 1. Diagram of general experimental arrangement.

Sampling of blood. 10 ml. glass syringes with metal nozzles were used. They were greased with liquid paraffin and their dead spaces filled with an isotonic saline solution containing heparin 7 mg/ml. to prevent clotting of the sample. A simple device enabled arterial and venous samples to be drawn simultaneously so that members of a pair were exactly matched in timing. The two syringes were held alongside one another in a stand (see Fig. 1), and a common handle which slipped over the end of both plungers allowed these to be withdrawn at the same rate. Polythene connectors joined the sampling syringes to the catheter or cannula in the vessel from which blood was being sampled, and the incorporation of three-way taps into the system allowed the dead spaces of the connectors to be cleared by a separate pair of syringes before the samples were drawn. About 8 ml. of blood was drawn into each sampling syringe, over a period of about 15 sec. During short experimental runs a pair of samples was drawn every minute, but they were taken less frequently in longer runs. Up to twelve pairs of samples were drawn per experiment.

Storage and analysis of blood samples. Blood samples were stored in the syringes in which they were drawn, and were insulated from the atmosphere by rubber tubing fitted over the nozzle of the syringe and plugged with a glass rod. A small amount of mercury was drawn into each syringe to facilitate mixing of the sample after storage, and syringes were kept at 0° C while the blood was awaiting analysis.

The 'total CO_2' content of each sample was measured by the van Slyke manometric method (Peters & van Slyke, 1932), and the radioactivity of this CO_2 was assayed by the method of van Slyke, Steele & Plazin (1951), using the gas-phase counting tubes of Bernstein & Ballentine (1950). Blood samples were analysed as soon as possible, and never later than 24 hr. after withdrawal from the animal. Analyses were carried out in the expected order of increasing radioactivity of the samples, to minimize 'memory' effects.

The greatest source of inaccuracy in the determinations of 'total CO_2' content was almost certainly in the measurement of 1 ml. of blood by pipette. Fortunately, errors in pipetting the blood sample would equally affect the determination of 'total CO_2' and of radioactivity, and would not therefore vitiate the determination of specific activity, this being a ratio of the two quantities measured in the same sample. Other errors in the 'total CO_2' determination did not exceed $\pm 1\%$, and the standard error of the radioactivity determinations lay between $\pm 1\%$ and $\pm 2\%$, depending on the counting rate of the particular sample.

Collection and analysis of expired CO_2. The respiratory circuit used is shown diagrammatically in Fig. 1. A steady stream of air was drawn past the tracheal cannula by an oil pump at the rate of about 1·5 l./min. This carried the expired air up the absorption tower, where the expired CO_2 was absorbed by two 60 ml. portions of 2N-NaOH containing about 2% of albumin to promote foaming (Kornberg *et al.* 1952); the towers containing the alkali were arranged in series. The valve system prevented leakage of expired CO_2 to the outside air, and the rubber bag prevented excessive fluctuations of pressure in the tracheal cannula. Each set of towers was used for a timed collection over a period of between 1 and 5 min if analysis was to be performed. At the end of the period the expired air was diverted to a different set of towers by three-way taps. The soda in the used towers was washed down into the volumetric flasks (see Fig. 1), made up to 200 ml. with CO_2-free water and kept stoppered while awaiting analysis. The 'total CO_2' content and radioactivity of the samples were measured by a method similar to that used for blood; a blank to take account of CO_2 absorbed from the atmosphere during manipulation of the alkali before and after the sampling period was determined by a run with no animal in the respiratory circuit.

Duration of experiments. Experiments usually lasted between 2 and 7 hr from the time of injection, by which time the blood CO_2 specific activity had fallen too low for accurate analysis. After the last blood samples had been drawn the animal was destroyed by an overdose of pentobarbitone and the position of any catheter used was confirmed by dissection.

RESULTS

A single tracer dose of sodium ^{14}C-bicarbonate (usually 10 μc) was injected intravenously into a different dog in each twenty-six experiments.

Arterial blood CO_2

Arterial blood was sampled serially in seventeen experiments. The specific activity of arterial blood CO_2 invariably followed the same time course. In the first 5 min after injection it declined very steeply, but the rate of fall was much reduced after 20 min, and in the final stages of the experiment the specific activity was falling very slowly. The measured specific activities in each experiment always fitted well to a smooth curve when plotted against time. Though the actual specific activity at any given interval after injection varied considerably from experiment to experiment, depending on the injected dose, the weight of the animal and other undefined factors, it was possible to superimpose the arterial CO_2 specific activity time curves from all experiments by multiplying all the measured specific activities in each experiment by a factor which made 10-min specific activity for that experiment arbitrarily equal to 100 (Fig. 2). The constancy of the shape of the curve suggests that the processes governing it are very constant from animal to animal, and the factors which determine this shape will be considered in the subsequent discussion.

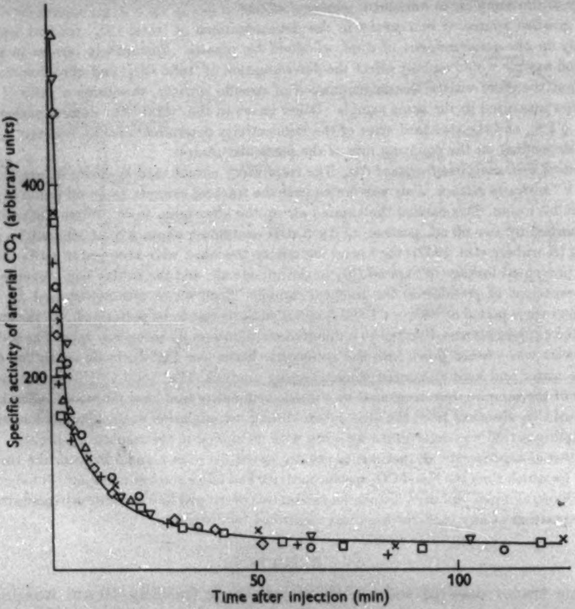

Fig. 2. Changes with time in specific activity of carbon dioxide in arterial blood following injection of ^{14}C-bicarbonate. The data are from seven different experiments, each represented by a different symbol (△, ▽, ◇, ×, □, + and ○) and they have been standardized by converting the observed 10-min value for specific activity (counts/min/μmole) to 100 and adjusting the other observed values by the same factor.

Expired CO_2. The specific activity of the CO_2 expired at any moment was the same as that of the CO_2 in the arterial blood at the same moment, except possibly in the period immediately following the injection. This is illustrated in Fig. 3. Since expired CO_2 had to be collected over a period of at least 1 min in order to make analysis reliable, the samples were necessarily integrated rather than instantaneous, and since the specific activity of the expired CO_2 was falling in a complex exponential fashion, the specific activity of the integrated sample would not equal that obtaining at the mid time of the collection period. However, when the collection periods were shortened to 1 min a good correspondence was shown between the specific activities of arterial and expired CO_2, as is evident from the points shown in Fig. 3. No points were obtained for the specific activity in the first half minute after injection.

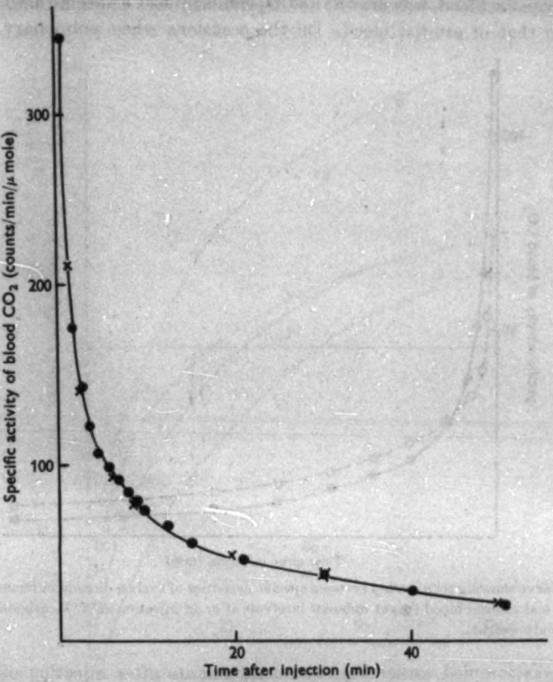

Fig. 3. Curve showing close correspondence of specific activities of carbon dioxide in arterial blood (●) and in expired air (×).

The total respiratory loss of $^{14}CO_2$ in the 2 hr period of the experiments was 50% or less of the injected dose, varying considerably from experiment to experiment. At the end of this period the rate of respiratory loss had fallen to about 3% of the injected dose per hour. No measurements were made of renal $^{14}CO_2$ loss.

Venous blood CO_2

Mixed venous blood CO_2. In two experiments 'mixed venous' blood was sampled from the pulmonary artery after ^{14}C-bicarbonate injection. The specific activity of mixed venous blood CO_2 was found to be identical with that of systemic arterial CO_2, within the limits of experimental error, after the third minute from the time of injection. The ratio of the specific activities was 1·01 ± 0·01, and 0·98 ± 0·01 (means and S.D.) in two separate experiments. In three other experiments right atrial blood, which is a less reliable sample of mixed venous blood, was drawn; its CO_2 similarly had a specific activity very close to that of arterial blood. On the occasions when pulmonary arterial blood was sampled earlier than the third minute after injection of isotope its CO_2 had a specific activity slightly lower than that of systemic arterial CO_2 at this time, the ratios being 0·89 and 0·92.

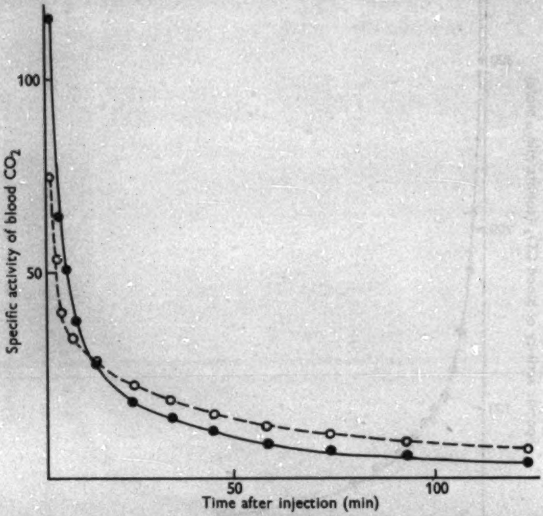

Fig. 4. Curve showing relationship between specific activities of carbon dioxide in femoral venous (○) and arterial blood (●) at different intervals after an injection of ^{14}C-bicarbonate (0·7 μc/kg body weight).

The cardiac output was calculated from the CO_2 figures according to the direct Fick procedure and was found to be 1 l./min, varying by only 0·1 l./min throughout the experiment, in two dogs weighing 8·2 and 8·9 kg, respectively.

Femoral venous blood CO_2. In eight experiments blood leaving a hind limb in a femoral vein was sampled simultaneously with arterial blood of the opposite limb at intervals after a single intravenous injection of ^{14}C-bicarbonate. In each experiment the specific activity of the CO_2 of the femoral venous blood showed the same general relationship to that of arterial blood. This relationship is illustrated in Fig. 4, from which it may be seen that for some minutes after injection the venous CO_2 had a far lower specific activity than the arterial. The two specific activities, which were both falling, converged and became equal at some time between 10 and 50 min after injection. The specific activity–time curves then crossed, and the venous CO_2 subsequently had a higher specific activity than the arterial for the remainder of the experiment. The relationship between specific activities is clearly shown by plotting the changes in the ratio of venous CO_2 specific activity to arterial CO_2 specific activity with time, using the figures for the pairs of simultaneous samples. Such a plot is shown in Fig. 5. This ratio (subsequently referred to as '*V*:*A* specific activity ratio') rose steadily for at least 100 min after injection. It began at or below 0·5 in most experiments. The cross-over time of the specific activity curves, when the ratio equalled 1·0, varied from 11 to 46 min after injection, the mean being 23 min.

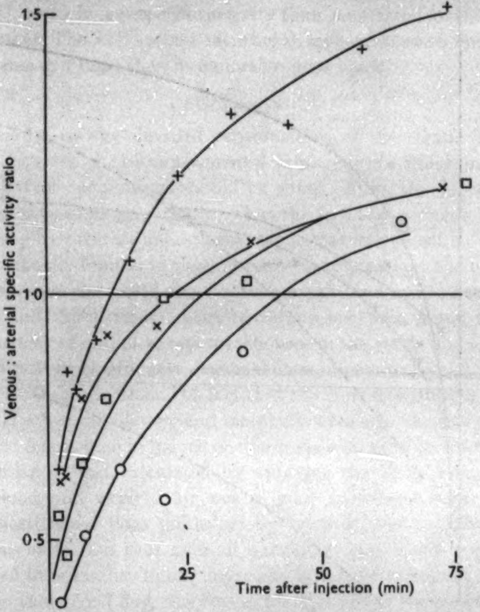

Fig. 5. Femoral venous: arterial ratios for specific activity of blood carbon dioxide at intervals following the injection of ^{14}C-bicarbonate. Each type of symbol refers to values from a different dog.

In four experiments femoral venous blood only was sampled, at rather shorter intervals than was practicable when arterial blood had also to be handled; this enabled the time course of changes in venous $^{14}CO_2$ to be defined more precisely. It was then found that the points fell less close to a smooth curve than did those for arterial specific activity, suggesting that there are factors affecting the rate of fall in specific activity of femoral venous CO_2 which are negligible, or become smoothed out, in the arterial CO_2 specific activity curve. Such variability in the venous values between different experiments precludes the construction of a composite curve by standardizing the specific activity scale, as was done successfully for the arterial values in Fig. 2. It was established, however, that the specific activity of CO_2 in femoral venous blood sampled simultaneously from opposite hind limbs of the same dog did not differ significantly in specimens taken more than 5 min from the

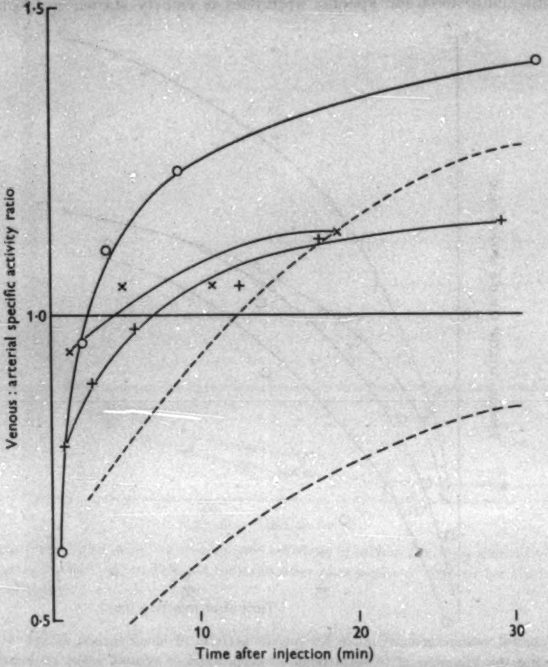

Fig. 6. Venous:arterial specific activity ratio for CO_2 of blood from external jugular vein after injection of ^{14}C-bicarbonate. Each type of symbol represents values for a different experiment. The broken lines show the time course of similar changes in blood from the femoral vein in other animals.

time of injection, so that the variability may be attributed to factors inherent in the animal rather than shortcomings in the sampling arrangements.

External jugular venous blood CO_2. External jugular venous blood CO_2 was sampled simultaneously with arterial blood at intervals after ^{14}C-bicarbonate injection in three experiments. The results, set out in Fig. 6, show that the specific activity of jugular venous blood CO_2 was initially below that of arterial CO_2, but the curves crossed in less than 10 min from the time of injection, and the venous CO_2 had a higher specific activity than the arterial for the remainder of the experiment. The $V:A$ specific ratio began higher (around 0·7) and reached unity sooner (mean 4 min) than in femoral venous blood.

DISCUSSION

Before proceeding to any detailed consideration of the significance of our results some features of the experimental arrangements which may restrict the applicability of our findings should be noted. First, the depth and type of anaesthetic have some importance. Thus the fact that animals were under moderately deep barbiturate anaesthesia means that movement in 'voluntary' muscles was virtually limited to those subserving respiration. The unconscious state may also have depressed the cerebral metabolism somewhat below the waking level and, furthermore, pentobarbitone has been found to produce certain aberrations of carbohydrate metabolism in the whole animal (Murphy & Young, 1932; A. St G. Huggett, personal communication) which might conceivably affect CO_2 production. The fact that the animals had been kept without food would affect circulatory and metabolic events in the splanchnic area and would also have some influence on the metabolic pattern elsewhere. The fact that cardiac output calculated by applying the Fick principle to our figures for blood and expired air was in good agreement with figures for resting unanaesthetized dogs published by Levy & Blalock (1937) affords, however, some indication that over-all circulatory conditions approximated resaonably well to normality in our dogs; and it may be worth emphasizing in this connexion that blood flow was free in the veins from which samples were taken.

From the standpoint of terminology it should be mentioned here that in the subsequent discussion bicarbonate and carbon dioxide are assumed to be freely interconvertible according to the equilibrium

$$HCO_3' \rightleftharpoons OH' + CO_2.$$

With these general points as a background, attention will be directed to certain special aspects of the results.

Mixing effects in relation to specific activity of blood CO_2

Radioactive bicarbonate injected rapidly into the venous side of the circulation in the form of what has been called a 'slug' will clearly undergo dilution when brought into contact with the non-radioactive bicarbonate already present in the animal's blood. Further, since the CO_2 of the blood has access to interstitial fluid, to intracellular fluid and to alveolar air, it is evident that mixing of radioactive with inactive molecules can also occur in all these situations. In addition to such dissemination amongst inactive molecules of CO_2 already present in the animal's body at the time of the injection, the radioactive molecules which are introduced will be further diluted from that instant onwards by inactive CO_2 produced in the course of oxidative metabolism. These processes will combine to bring about variations in the proportion of radioactive to total CO_2 in the arterial blood leaving the lungs, and these variations may extend over a considerable period of time, as will be evident in curves, such as that seen in Fig. 2, which relate changes in specific activity of arterial CO_2 to time.

The fact that such changes are so consistent in all experiments, as demonstrated by the very slight scatter of the points in Fig. 2, prompts some inquiry into the factors which determine this sequence of events. One possible approach in such an inquiry consists in a formal mathematical analysis of the curve, for which purpose the procedure of successive extrapolations and subtractions has been frequently adopted. This type of mathematical treatment was apparently developed by Bateman (1910) and used in the analysis of the rates of radioactive decay (see Rutherford, Chadwick & Ellis, 1930) but it has lately been widely favoured in the study of biological systems (Solomon, 1949). The application of this form of analysis to the arterial specific activity–time curve for one of our experiments is shown in Fig. 7. In this graph some of the values from Fig. 2 have been replotted on semi-logarithmic co-ordinates and the nearly linear terminal portion has been extrapolated back to the ordinate axis. On subtracting the co-ordinates of points on the linear extrapolation from those on the original curve a residual curve can be constructed. Repetition of the extrapolation followed by further subtraction produces a new curve and so on, as indicated in Fig. 7; four exponential components at least can be identified with half-times of approximately $\frac{1}{2}$, 2, 10 and 59 min, respectively. However, such an analysis does not, of itself, provide any information as to the physical nature of the rate-processes which lead to the observed results, and in fact, the usefulness of the method becomes rather limited when more than three components are implicated (Solomon, 1949; Sacks, 1953).

Nevertheless, certain interesting similarities are revealed when the characteristics of the specific activity–time curve for arterial CO_2 are compared with those of curves showing the time course of mixing of other materials introduced into the circulation. In the case of Evans Blue, which, at least initially, does not leave the vascular system, a characteristic 'dilution curve' can be distinguished in the arterial blood (Newman, Merrell, Genecin, Monge, Milnor & MacKeever, 1951; see also, Hamilton, Moore, Kinsman & Spurling, 1928).

This curve, after a primary hump and possibly a series of secondary humps, assumes within about 30 sec a flat horizontal contour. Similar curves can be produced by other materials such as ^{32}P-labelled red cells which remain within the vascular compartment (Groom, 1954). In the case of ^{24}Na (Gellhorn, Merrell & Rankin, 1944) which mingles with the unlabelled Na of the extracellular fluid and of D_2O (Edelman, 1952) which can exchange with intracellular water as well as extracellular, the curves show a markedly negative slope for many minutes following injection. The curves for ^{24}Na and D_2O bear a marked similarity to the curve for arterial $^{14}CO_2$; so also does a curve plotted from Gaudino's (1949) data on the equilibration of inulin through the extracellular fluid.

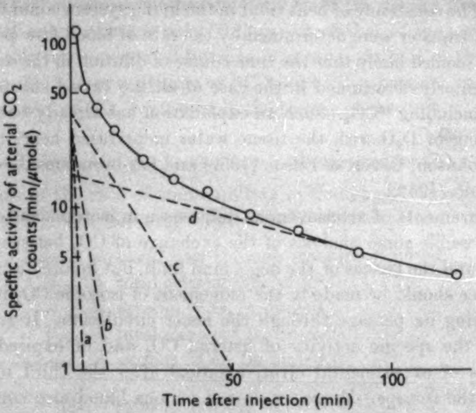

Fig. 7. Resolution of arterial specific activity–time curve on semi-logarithmic co-ordinates by successive extrapolations. The exponential components labelled *a*, *b*, *c*, and *d* gave calculated half-times of decline of 0·5, 2·2, 9·5 and 58·5 min respectively.

Gellhorn *et al.* (1944) quote half-times of 0·7 and 7·3 min for two of the components which can be extracted from their arterial specific activity curves for ^{24}Na, and Edelman (1952) obtained components with half-times of 1·5 and 12·8 min from his curve for arterial D_2O. Gaudino's results for inulin can be fitted by an equation which includes three terms giving half-times of 1·9, 7·3 and 54 min, although he himself employs an equation containing only two terms. The similarity between the fastest components for ^{24}Na and $^{14}CO_2$ which, according to Pappenheimer (1953) traverse capillary walls by different paths, namely the intracellular pores and the cell bodies respectively, is particularly remarkable.

The fact that this heterogeneous group of substances, which differ not only chemically but also in their ability to penetrate the fluid compartments of the body, all exhibit a similar behaviour in respect of their rate of dilution in the blood stream suggests that this behaviour is determined by some factor other than the boundaries of their volumes of dilution and their rate of random dispersal within this volume. The situation is, in fact, closely comparable to that described by Jones (1950) in his studies of the rate of transfer of inert gases into and out of living tissues; these appeared to be constant for a series of gases whose behaviour in penetrating a gelatin membrane showed considerable differences. The constancy of behaviour in the living system could be explained if the rate of transfer were determined by the rate of blood flow per unit mass of tissue. It seemed likely that the time course of dilution in the arterial blood might be similarly determined in the case of all the varied substances so far considered, including $^{14}CO_2$. Such an explanation has already been advanced for the mixing of D_2O with the tissue water in perfused heart and skeletal muscle by Johnson, Cavert & Lifson (1952) and has been considered for $^{14}CO_2$ by Drury *et al.* (1956).

Our measurements of arteriovenous differences in isotope content of blood CO_2 make possible some analysis of the exchange of CO_2 between the circulating blood and the tissues of the dog's hind limb, but before discussing these brief reference should be made to the movement of isotopic CO_2 into and out of blood during its passage through the lesser circulation. It is clear from Fig. 3 that the specific activity of arterial CO_2 and of expired CO_2 were, within limits of experimental error, identical after the third minute after injection of the isotope. Moreover, mixed venous blood also contained CO_2 the specific activity of which was identical with that of expired CO_2 and arterial CO_2. It is true that, as noted above in the section on Results (p. 474), there is some suggestion that the mixed venous blood might contain CO_2 of somewhat different specific activity from that of arterial CO_2 during the first few minutes of an experiment where sampling was begun very early, but this would be readily explained by the initial heavy labelling of alveolar CO_2 by the 'slug' of injected isotope before dilution in the blood stream had proceeded very far. (This corresponds to the primary hump in an arterial dye-dilution curve). If such heavy labelling took place during the first passage of isotope-carrying blood, the blood on successive circuits would exchange $^{14}CO_2$ with it until isotopic equilibrium had been attained. At subsequent stages, that is after about 4 min in our experiments, no demonstrable changes in the specific activity of its CO_2 occurred during the passage of blood through the pulmonary circuit.

Arteriovenous differences in the hind limb

In striking contrast to the situation in the pulmonary vascular bed there was a considerable difference in specific activity of the CO_2 contained in the blood entering and that leaving the main vessels of the hind limb. The fact that an increase in total CO_2 content occurred during the passage of blood through the limb raises the possibility of dilution of the arterial $^{14}CO_2$ by $^{12}CO_2$ derived from oxidation of unlabelled substrates. However, calculation of the possible contribution of such $^{12}CO_2$ to the observed arteriovenous difference in specific activity shows that it could only account for a small fraction of the change which actually took place. Thus, since the total CO_2 content of femoral vein blood was only 14 % greater than that of arterial blood, whereas in many instances the specific activity of the venous CO_2 was in the early stages of an experiment only 50 % or less of that of the arterial CO_2, it follows that the arteriovenous difference in specific activity of the blood CO_2 must have resulted from exchange of $^{14}CO_2$ and $^{12}CO_2$ between the blood and a pre-existing pool of CO_2 contained in the tissues of the limb.

Calculation of transcapillary exchange rate for CO_2

An estimate of the rate of transcapillary exchange of CO_2 may be derived from our figures in the following way:

Let B = blood flow through the limb (l./min),
A_{CO_2} = total arterial CO_2 (m-mole/l.),
a_{CO_2} = arterial $^{14}CO_2$ (m-mole/l.),
V_{CO_2} = total venous CO_2 (m-mole/l.),
v_{CO_2} = venous $^{14}CO_2$ (m-mole/l.),

$$Sa = \text{specific activity of arterial } CO_2 = \frac{a_{CO_2}}{A_{CO_2}}, \text{ and}$$

$$Sv = \text{specific activity of venous } CO_2 = \frac{v_{CO_2}}{V_{CO_2}}.$$

If exchange takes place in the manner indicated in Fig. 8, then y m-moles of CO_2 having the same specific activity as that in capillary blood (Sc) pass into the tissue each minute, and x m-moles of CO_2 of the same specific activity as tissue CO_2 (St) pass out of the tissue into the blood. The net entry of $^{14}CO_2$ into the new tissue under such conditions will then be

$$ySc - xSt \text{ m-moles/min.}$$

This will also be given by $B(a_{CO_2} - v_{CO_2})$. (1)

Hence $$ySc - xSt = B(a_{CO_2} - v_{CO_2}).$$

Now during the time of the first passage of blood containing $^{14}CO_2$ through the tissue, the specific activity of the CO_2 in the tissue may be regarded as being close to zero, and equation (1) then takes the simpler form

$$ySc = B(a_{CO_2} - v_{CO_2}). \tag{2}$$

The solution of this equation depends upon the value assigned to Sc.

A maximum value of Sc would be set by putting it equal to the specific activity of arterial CO_2, that is to Sa, in which case equation (2) becomes

$$ySa = B(a_{CO_2} - v_{CO_2}), \tag{3}$$

whence

$$\frac{y}{B} = \frac{(a_{CO_2} - v_{CO_2})}{Sa} \tag{4}$$

or, substituting from the relations

$$Sa = \frac{a_{CO_2}}{A_{CO_2}} \quad \text{and} \quad Sv = \frac{v_{CO_2}}{V_{CO_2}},$$

we have

$$\frac{y}{B} = \frac{Sa\,.\,A_{CO_2} - Sv\,.\,V_{CO_2}}{Sa}$$

$$= A_{CO_2} - V_{CO_2}\left(\frac{Sv}{Sa}\right). \tag{5}$$

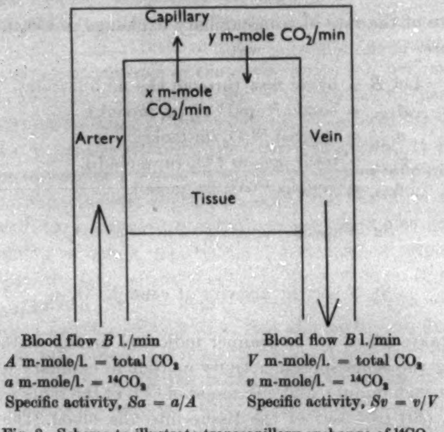
Capillary
y m-mole CO₂/min
x m-mole CO₂/min
Artery
Vein
Tissue
Blood flow B l./min
A m-mole/l. = total CO₂
a m-mole/l. = ¹⁴CO₂
Specific activity, Sa = a/A
Blood flow B l./min
V m-mole/l. = total CO₂
v m-mole/l. = ¹⁴CO₂
Specific activity, Sv = v/V

Fig. 8. Scheme to illustrate transcapillary exchange of $^{14}CO_2$

Now, since the term y/B has the dimensions of m-moles/l. it can be expressed as a percentage of A_{CO_2}; but in order to obtain a numerical solution to equation (5) it is necessary to know Sv/Sa at zero time. This can be obtained, though not with any great precision, by extrapolating the curves in Fig. 5 back to zero on the time axis. The validity of this extrapolation is adversely affected by the fact that sampling began only after the passage of the bicarbonate 'slug', which may have raised the specific activity of tissue CO_2 quite sharply. However, an error from this source would tend to underestimate y/B, as would the assumption of a specific activity of capillary CO_2 identical with that of arterial CO_2. For these two reasons our estimate of the exchange rate from equation (5) will give a minimum value. This in fact turns out to lie between 35 and 58% in the experiments on which the curves in Fig. 5 are based, the mean being 48%. If Barcroft's expression (1934) for the relation of capillary pO_2 to arterial pO_2 be assumed to define also the relation between arterial $^{14}CO_2$ and capillary $^{14}CO_2$, the estimates of exchange rate rise to 50–90% with a mean of 76%.

Since the rate of transcapillary exchange of CO_2 is such that not less than a third of the arterial CO_2 is involved in this process during its passage through the limb, it is interesting to consider first why the venous specific activity does not become equal to that of arterial blood until for what seems *a priori* a relatively long time, namely some 20 min from the giving of the injection. It is also important to attempt an explanation of the occurrence of a phase extending over a considerable period of time later in the experiments, when the specific activity of venous CO_2 exceeds that of arterial.

An explanation of the time required for the two specific activities to become identical would readily be provided if the limiting factor in the exchange process as a whole were the blood perfusion rate in the tissue. Such was found to be the case in the exchange of D_2O in resting muscle studied by Johnson *et al.* (1952). If the rate of exchange of $^{14}CO_2$ in the limb is assumed to be blood-flow limited we may picture the constituent tissues as containing two pools of CO_2; one of them will be confined to the tissue fluid in close apposition to the blood capillaries, and will exchange any isotopic CO_2 which it may contain very rapidly with the blood CO_2; a second pool, more remote from the blood vessels than the first, is pictured as exchanging its CO_2 more slowly with that of the first pool. Such a concept would be consistent with the finding of Drury *et al.* (1956) that the specific activity of the total CO_2 in muscle remains below that of arterial blood in rabbits for 15 min after an injection of ^{14}C-bicarbonate.

In seeking to account for the phase in the specific activity ratio–time curve (Fig. 5) when the venous values exceed those of the arterial CO_2, an obvious factor to be considered is the contribution of metabolic CO_2 generated in the body by the oxidation of unlabelled substances. The existence of an arterio-venous difference in total CO_2 content across the limb proves that such metabolic CO_2 is being produced in the limb, and it may be confidently assumed that this would be of low specific activity, since very little oxidizable material could have been heavily labelled by CO_2 incorporation in the time under consideration (Steele, 1955). Since then the outflow from the limb is contributing, to the mixed venous blood reaching the heart, CO_2 of greater specific activity than that found in arterial blood (which in turn is the same as that in mixed venous blood), it seems necessary to infer that other tissues must be contributing unlabelled CO_2 to the mixed venous blood at a much faster rate than tissues of the limb, in order to explain the falling specific activity of arterial CO_2.

These tissues are in all probability those which also pour labelled CO_2 rapidly into the blood when radioactive glucose is administered to a dog (Coxon & Robinson, 1959) and include liver, brain and kidneys. These tissues are to be regarded as having a high blood flow in relation to the CO_2 pool, and, in conformity with such an idea, Drury *et al.* (1956) found that the CO_2 of liver attained a specific activity equal to 88% of that of arterial CO_2 within 1 min of injecting ^{14}C-bicarbonate; however, it never actually attained the same level as that of arterial blood and this was attributed to a high rate of production of metabolic $^{12}CO_2$ in the organ. The absence of a similar phase where isotope concentration in venous blood exceeds that in arterial blood in Edelman's (1952) experiments with D_2O may readily be explained as due to the great size of the body pool of H_2O, which would obscure any difference of the magnitude seen with $^{14}CO_2$.

Contribution of different tissues of limb to CO_2 pool

Drury *et al.* (1956) concluded that more than 80% of the extra-vascular CO_2 which mixed relatively rapidly with blood CO_2 was to be found in muscle and skin. Resting muscle would be expected to have a relatively low blood-perfusion rate (Krogh, 1922) and the skin, in the absence of vasodilatation for cooling purposes, would similarly not have a particularly rapid blood flow. Thus the rate of attainment of isotopic equilibrium in the muscle and skin of a resting limb might well be relatively slow compared with that in internal organs such as the liver. The contribution of bone to the dilution of injected ^{14}C-bicarbonate in the limb is probably not very great in short-term experiments but would be expected to assume greater significance in more prolonged studies. Thus Kornberg & Krebs (1952) found that some 7% of an injected dose of $NaH^{14}CO_3$ was retained in the skeleton 4 hr after injection. But since bone (in man) receives only about 1% of the cardiac output, even if the blood leaving bone were completely free of $^{14}CO_2$ it may be calculated that the rate of fall of specific activity of arterial CO_2 which could be brought about in this way would have a half-time of 100 min, which is far slower than any identifiable component in our curves. The effect of clearance of $^{14}CO_2$ by lymphatic flow from a resting limb is also likely to be negligibly small since, according to Yoffey & Courtice (1956), the flow of lymph in the thoracic duct is less than 1 ml./min in dogs of the size used by us.

SUMMARY

1. The time course of changes in specific activity of carbon dioxide in expired air and in arterial, mixed (central) venous and peripheral venous blood has been examined after the injection into dogs of ^{14}C-bicarbonate.

2. At times later than 3 min after the injection the specific activity of the

carbon dioxide was found to be the same in arterial and mixed venous blood and in expired air.

3. For a variable period (the mean duration being 23 min), the specific activity of the CO_2 in peripheral (femoral) venous blood was less than that in arterial blood, but subsequently exceeded it.

4. An estimate is made of the rate of transcapillary exchange of $^{14}CO_2$ in a dog's limb and the factors determining this rate are discussed. It seems likely that the process is blood-flow limited.

One of us (R.V.C.) wishes to acknowledge his indebtedness to Drs D. D. van Slyke, R. Steele and F. M. Sinex for valuable advice and to Mr J. Plazin and Miss J. MacMullen for expert technical help in the initial stages of this investigation. We are deeply indebted to Dr R. H. Kay for his advice on the assembly and maintenance of the counting apparatus. We are also grateful to Messrs Eli Lilly for a grant towards the cost of apparatus and to the Christopher Welch Fund of Oxford University and the Governing Body of Christ Church for a Scholarship and Lectureship respectively, held by R.J.R. during the course of the work.

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MOVEMENTS OF RADIOACTIVE CARBON DIOXIDE WITHIN THE ANIMAL BODY DURING OXIDATION OF ^{14}C-LABELLED SUBSTANCES

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In the preceding paper (Coxon & Robinson, 1959) we have described a study of the transport in dogs of radioactive carbon dioxide into and out of different regions of the body following a single intravenous injection of radioactive bicarbonate. The present report is concerned with a similar investigation, on a regional basis, of the movements of isotopic carbon dioxide following the administration of radioactive glucose to dogs and monkeys. In the latter case the situation is complicated by comparison with that which arises after the injection of isotopic bicarbonate in that radioactive carbon dioxide is endogenously generated from the glucose over a considerable period of time. As in the simpler case of exogenous labelling of the animal's body-content of carbon dioxide we have measured the arteriovenous differences in specific activity of the blood carbon dioxide in a number of vascular territories at varying intervals of time after a single intravenous injection of ^{14}C-glucose (or other oxidizable substance). The results of these measurements will be discussed in terms of the differing characteristics of local pools of carbon dioxide which they reveal; and the effect of these heterogeneous pools upon the time course of changes in the specific activity of carbon dioxide in the arterial blood and expired air will be considered with particular reference to the validity of model systems employed by other authors in the interpretation of such changes. A brief note on some of the results has already appeared (Robinson & Coxon, 1957).

METHODS

The general arrangements for collecting and analysing samples of blood and expired air were identical with those previously described (Coxon & Robinson, 1959). In the majority of experiments the animals were under pentobarbitone anaesthesia but in one instance thiopentone (which is said to produce less change in blood sugar levels) was substituted without, however, affecting the results.

Uniformly labelled glucose, obtained from the Radiochemical Centre, Amersham, was injected intravenously in tracer quantities. The dose given was approximately 1 μc radioactivity/kg body

weight, contained in 1 mg of sugar. The animals used comprised fifteen mongrel dogs weighing between 10 and 30 kg and one rhesus and one pigtail monkey, weighing 2·5 and 4·5 kg, respectively. (The doses given to the monkeys are indicated with the results of the individual experiments.) Carboxyl-labelled acetate was given to three dogs in a dose of either 1 or 2 μc/kg, approximately, and carboxyl-labelled palmitate was administered to one dog in a dose of 5 μc/kg body weight.

Arterial blood was sampled in all experiments; details of the method of obtaining venous blood from different regions are mentioned with the results. Catheterization of deeply situated veins was carried out under fluoroscopic control. Coincident timing of the arterial and venous sampling was ensured by using coupled syringes.

RESULTS

Arterial blood

The time course of changes in the specific activity of the carbon dioxide (SA_{CO_2}) in arterial blood is illustrated in Fig. 1, where the results from four animals are plotted. The general form of this curve was similar in all experiments but there were variations in the detailed contour which made it impossible to combine the results of all experiments in a single curve by simply adjusting the scale, as was done for some earlier experiments when $H^{14}CO_3^-$ had been injected (Coxon & Robinson, 1959). This indicates that, as might be expected, there are more contributory variables when ^{14}C is introduced in the form of glucose. The time of peak specific activity had a mean value of 62 min in the present experiments but there was considerable scatter, as may be seen from the individual graphs.

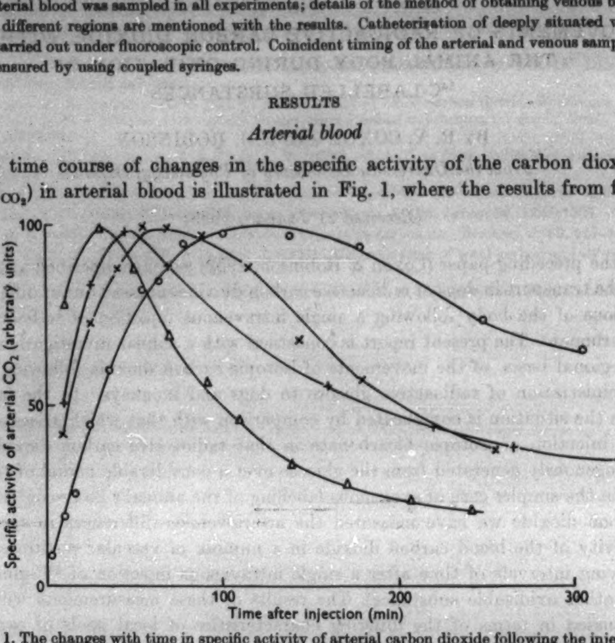

Fig. 1. The changes with time in specific activity of arterial carbon dioxide following the injection of ^{14}C-glucose. Each curve is from a different experiment and the peak specific activity is in each case put equal to 100, the other values being then adjusted by a corresponding factor.

Expired air. In the seven samples in which it was determined the specific activity of the carbon dioxide (SA_{CO_2}) in expired air gave a value very close to that of blood. The ratio of the specific activities of arterial and expired CO_2 varied between 0·96 and 1·05 with a mean of 1·00 in the 24 determinations made.

Accurate measurement of rate of loss of the injected ^{14}C as $^{14}CO_2$ was not a primary aim of these experiments, and the periods of collection of expired CO_2 were short. However, it was possible to calculate the $^{14}CO_2$ output in any given period from the mean rate of total CO_2 production and a mean value for expired CO_2 specific activity during the period, obtained by integration of the specific activity curve. In this way it was calculated that the fraction of the injected ^{14}C which was expired in the first hour after injection varied from 5 to 17% of the injected dose, being higher in experiments where the peak specific activity of arterial CO_2 was reached early. Between 24 and 38% of the injected ^{14}C was expired in the first 3 hr after the injection.

Venous blood

Mixed venous blood was sampled from the pulmonary artery on seven occasions during one experiment and the mean ratio of the specific activity of its CO_2 to that in arterial blood was found to be $0{\cdot}98 \pm 0{\cdot}03$ in the seven pairs of samples.

Femoral and lumbar venous blood. Femoral venous blood was sampled simultaneously with arterial blood in five experiments. Figure 2 shows the changes in specific activity of the CO_2 with time, in arterial and femoral venous blood, in a typical experiment. It will be seen that the venous CO_2 gave a specific activity–time curve of similar general shape to that in arterial blood, but the venous SA remained below the arterial for the first 2–3 hr after the injection of labelled glucose. If the ratio of venous to arterial specific activity (which we shall call the $V:A$ ratio) be plotted against time, then curves of the type shown in Fig. 3 are obtained. The results of the three experiments on which the femoral curves in Fig. 3 are based show that the ratio begins with a value of about 0·5 and rises slowly to unity over a period of 3 hr or more. Ratios appreciably in excess of unity were not encountered, as had happened in earlier experiments with bicarbonate.

Since the ratio of venous total CO_2 to arterial total CO_2 had a mean value of only 1·15, it is obvious that the low $V:A$ ratio for specific activity found in the early stages of our experiments cannot be attributed simply to the addition of inactive CO_2 to the blood during its passage through the limb.

In one experiment a large vein draining the lumbar muscles (as was found at autopsy) was mistaken on the X-ray screen for the renal vein and was catheterized. Blood from this gave similar $V:A$ ratios to those found in femoral venous blood (see Fig. 3).

Hepatic venous blood. In two experiments arterial and hepatic venous blood was sampled simultaneously after ^{14}C-glucose injection. The hepatic

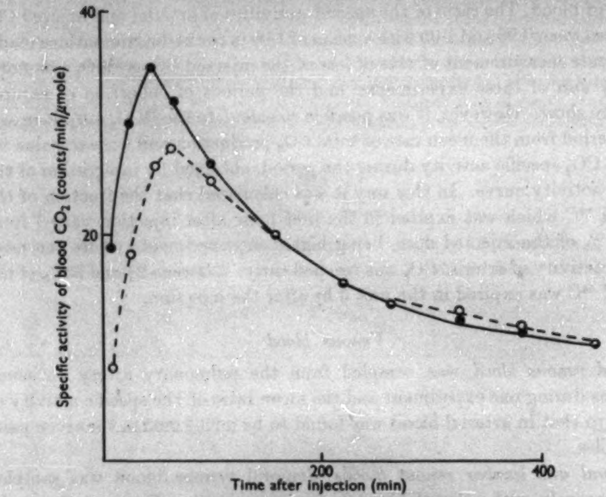

Fig. 2. The changes in specific activity of the CO_2 with time in femoral venous (○) compared with arterial (●) blood in a typical experiment.

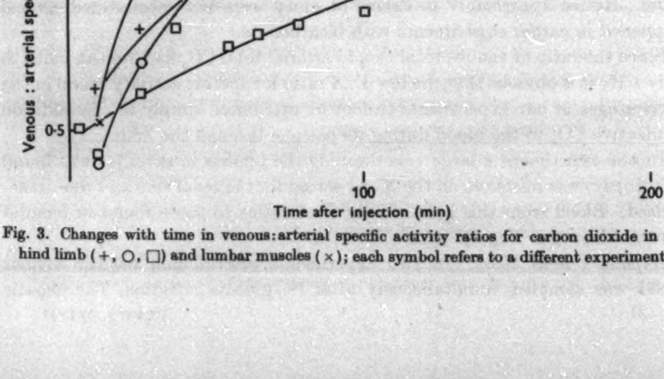

Fig. 3. Changes with time in venous: arterial specific activity ratios for carbon dioxide in the hind limb (+, ○, □) and lumbar muscles (×); each symbol refers to a different experiment.

venous blood CO_2 had a higher specific activity than the arterial for about 150 min after the injection. The full results for one such experiment are given in Table 1 and it will be noted that the $V:A$ specific activity ratio was initially between 1·1 and 1·2. The second experiment gave a more erratic series of values but the general course of events was the same, as is shown in Fig. 4.

TABLE 1. Hepatic arteriovenous differences in specific activity of blood CO_2 after ^{14}C-glucose I.V. injection

Time after injection (min)	Blood CO_2 specific activity: Arterial (counts/min/ µmole)	Venous (counts/min/ µmole)	$V:A$ specific activity ratio
9·2	6·3	7·4	1·17
24·6	13·0	15·0	1·15
39·5	14·7	17·0	1·16
61·2	14·7	15·8	1·07
80·4	12·5	13·1	1·05
100·4	11·4	12·0	1·05
122·3	9·3	10·0	1·07
147·5	7·6	7·8	1·02
171·1	6·7	6·6	0·98
197·6	5·7	5·6	0·97

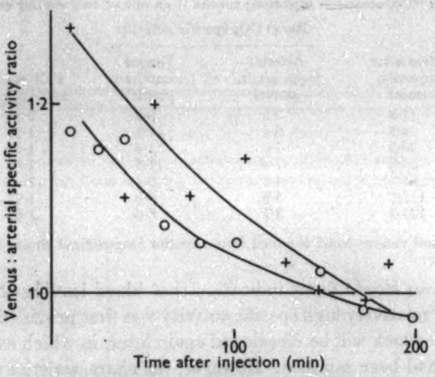

Fig. 4. Changes with time in hepatic venous: arterial specific activity ratio, following I.V. injection of ^{14}C-glucose, in two experiments.

Renal venous blood. Renal venous blood was sampled in two experiments. Rather irregular specific activity curves were obtained. The renal venous CO_2 clearly had a specific activity higher than the arterial CO_2 in the first 30 min after injection, the initial $V:A$ specific activity ratio being about 1·2; but the two specific activities become equal more quickly than in any other region, as may be seen from Table 2, which gives the results of one such experiment. The high blood-perfusion rate of the kidney makes studies of arteriovenous differences more difficult here than in other regions. In both our experiments the catheter was in the left renal vein, so that some spermatic and ovarian venous blood may have been included in the samples. The para-amino hippurate extraction ratio, however, was of the order of 75%, suggesting that most of the blood had flowed through actively functioning renal tissue.

Table 2. Renal arteriovenous differences in specific activity of blood CO_2 after ^{14}C-glucose i.v. injection; results from one of two similar expts.

Time after injection (min)	Blood CO_2 specific activity: Arterial (counts/min/ μmole)	Blood CO_2 specific activity: Venous (counts/min/ μmole)	$V:A$ specific activity ratio
13·0	12·8	14·8	1·16
30·4	18·4	19·0	1·03
45·9	18·2	19·3	1·06
66·5	19·0	19·0	1·00
90·5	18·8	18·8	1·00
121·2	16·5	15·8	0·96
152·5	14·3	14·3	1·00
186·5	13·2	12·7	0·96

Table 3. Cerebral arteriovenous differences in specific activity of blood CO_2 after ^{14}C-glucose i.v. injection; results from one of two similar expts.

Time after injection (min)	Blood CO_2 specific activity: Arterial (counts/min/ μmole)	Blood CO_2 specific activity: Venous (counts/min/ μmole)	$V:A$ specific activity ratio
11·3	3·5	8·2	2·34
24·6	6·1	11·1	1·82
39·3	7·0	11·4	1·63
55·9	7·0	11·5	1·64
94·5	4·8	9·1	1·90
112·5	3·9	6·9	1·77
137·3	3·7	6·0	1·62

Cerebral venous blood sampled from superior longitudinal sinus of dog.

Cerebral venous blood. Some indication that blood leaving the brain might contain CO_2 of relatively high specific activity was first provided by the results of experiments which will be mentioned again later, in which external jugular venous blood had been sampled. However, the characteristics of the cerebral venous blood were most clearly shown in a dog experiment in which samples were taken from the superior sagittal sinus by means of a needle inserted through a burr hole previously made in the skull. The results of this experiment are given in full in Table 3.

Two experiments on monkeys, in which cerebral venous blood was sampled from the internal jugular vein, confirmed in a qualitative fashion the results obtained in the dog. In the monkey, as in man, according to Kety & Schmidt (1945), the internal jugular vein contains a higher proportion of blood of cerebral origin than in the dog. Plots of the $V:A$ ratios in the dog and in one of the monkey experiments are shown in Fig. 5.

Simultaneous sampling from more than one region

In one experiment on a monkey, blood samples were taken simultaneously from internal jugular and femoral veins and from an artery. The relationship between the specific activities of CO_2 in these three situations is shown in Fig. 6. The nature of this relationship is entirely consistent with what would be expected from a survey of the combined results of dog experiments in which different regions were studied in different animals.

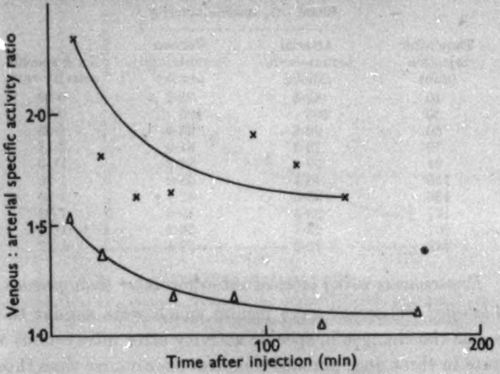

Fig. 5. Changes with time in the venous: arterial ratios of specific activity of carbon dioxide in blood traversing the brain following the intravenous injection of ^{14}C-glucose in a dog (×) and a monkey (△).

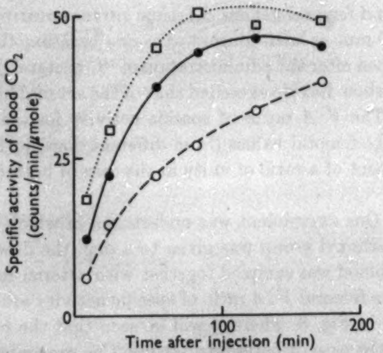

Fig. 6. Changes with time in specific activity of carbon dioxide in arterial (●), internal jugular (□) and femoral venous (○) blood, following the intravenous injection of 20 μc of ^{14}C-glucose into a pigtail monkey weighing 4·5 kg.

The results of studies on blood from the external jugular vein of dogs, such as those set out in Table 4, conform with what might be expected of an effluent which represented a mixture of blood from muscular and supporting tissues (having the same general characteristics as those of the hind limb) with blood from cerebral tissue. This is in agreement with the anatomical distribution of the tributaries of the external jugular vein in the dog.

TABLE 4. Arteriovenous differences in specific activity of blood CO_2 in the external jugular blood of a dog

Time after injection (min)	Blood CO_2 specific activity: Arterial (counts/min/ μmole)	Blood CO_2 specific activity: Venous (counts/min/ μmole)	$V:A$ specific activity ratio
10	82·4	73·2	0·89
30	105	103	0·98
50	92·8	95·4	1·03
70	72·9	81·0	1·11
91	59·4	65·2	1·10
110	48·4	58·0	1·20
136	35·5	42·1	1·19
171	29·5	40·6	1·38
207	24·8	34·2	1·38
242	22·2	28·1	1·27

Experiments using labelled substrates other than glucose

Carboxyl-labelled ^{14}C-acetate gave results which were similar to those with ^{14}C-glucose, but the changes in specific activity after intravenous administration of acetate in three dogs pursued a shorter time course than those resulting from the administration of ^{14}C-glucose. As is illustrated in Fig. 7, the peak specific activity in arterial blood occurred very much earlier than with glucose —at 6 min as against a mean value for glucose of 62 min. Moreover, the cross-over of arterial and femoral venous *SA*–time curves occurred at about 80 min instead of at 150 min as with glucose. On one occasion the hepatic venous blood was examined after the administration of ^{14}C-acetate. In this the specific activity of the carbon dioxide exceeded that of the arterial blood in the sample taken at 5 min. The $V:A$ ratios of specific activity for the liver effluent are compared with the femoral values (from different dogs) in Fig. 8. This shows the early attainment of a ratio of unity in the case of both femoral blood and hepatic blood.

^{14}C-palmitate. One experiment was undertaken in which sodium palmitate labelled in the carboxyl group was given to a dog, the dose being 4·5 μc/kg. Femoral venous blood was sampled together with arterial in this experiment. The values for the femoral $V:A$ ratio of specific activity are plotted alongside those for acetate in Fig. 8, where it will be seen that the ratio attains unity rather sooner in the case of palmitate. It must be remembered, of course, in this connexion that the ^{14}C in the carboxyl-labelled palmitate molecule would be found in the first 2-carbon fragment to be split off and oxidized.

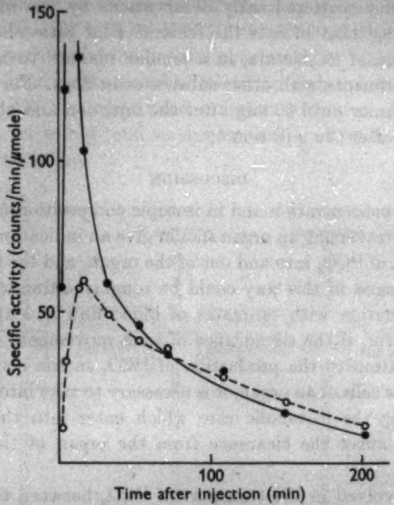

Fig. 7. Changes with time in specific activity of the carbon dioxide in arterial (●) and femoral venous (○) blood following intravenous ^{14}C-acetate in 3 dogs.

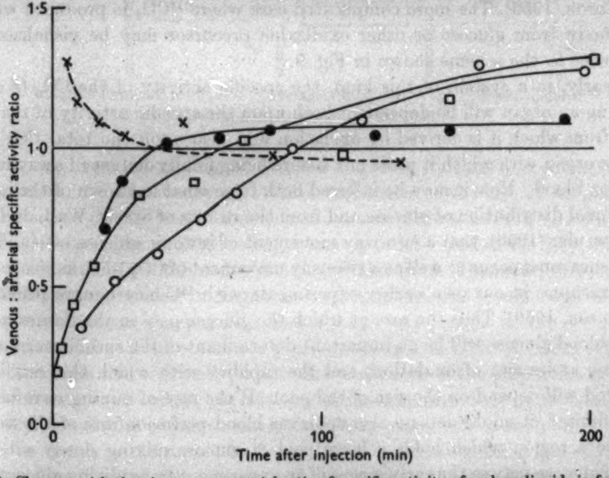

Fig. 8. Changes with time in venous: arterial ratio of specific activity of carbon dioxide in femoral venous (□, ○) and hepatic venous (×) blood following intravenous injection of ^{14}C-acetate, and in femoral venous blood following intravenous injection of ^{14}C-palmitate (●). Each curve is from a different dog.

^{14}C-lactate. Some scattered early observations by one of us (Coxon, unpublished) revealed that in cats the femoral *V*:*A* ratio changed with time after the injection of ^{14}C-lactate, in a similar manner to that observed in the present experiments with other substrates in dogs. For example, it was generally below unity until 30 min after the injection and above unity when determined 2 hr after the injection.

DISCUSSION

Changes in total concentration and in isotopic composition undergone by the CO_2 of the blood traversing an organ should give an indication of the direction of net movement of $^{14}CO_2$ into and out of the organ, and the actual quantities of isotope exchanged in this way could be roughly estimated by using such values in combination with estimates of blood flow and applying the Fick principle. However, if the significance of such movements of $^{14}CO_2$ is to be evaluated in relation to the production of $^{14}CO_2$ in the course of oxidative metabolism in the cells of an organ, it is necessary to take into account several factors other than the metabolic rate which enter into the over-all chain of events which affect the clearance from the organ of the CO_2 produced in it.

The factors involved in the transport of $^{14}CO_2$ between tissues and blood after the isotope has been injected in the form of a single rather massive dose of radioactive bicarbonate have been considered in the previous paper (Coxon & Robinson, 1959). The more complicated case where $^{14}CO_2$ is produced within the body from glucose or other oxidizable precursor may be visualized by reference to the scheme shown in Fig. 9.

Clearly, in a system of this kind, the specific activity of the CO_2 (SA_{CO_2}) leaving an organ will be dependent both upon the specific activity of the carbon from which it is derived by oxidation and also upon the total CO_2 pool in the organ with which it must mix before being finally conveyed away in the venous blood. Now it may be inferred both from what is known of the intracorporeal distribution of glucose and from the results of Steele, Wall, de Bodo & Altszuler (1956), that a two-way movement of isotopic glucose into and out of tissues must occur as well as a two-way movement of CO_2, such as was found, for example, in our own earlier experiments with ^{14}C-bicarbonate (Coxon & Robinson, 1959). Thus the rate at which the glucose pool in the tissues mixes with blood glucose will be an important determinant of the specific activity of glucose at its site of oxidation, and the rapidity with which this mixing is effected will depend on the size of the pool. If the rate of mixing were blood-flow-limited, it might depend also upon the blood-perfusion rate of the tissue. Hence a region which holds a large pool of glucose mixing slowly with the blood glucose may in the early stages of an experiment be oxidizing glucose of a lower specific activity than that of the blood glucose, yet later on the specific activity of the glucose at the oxidation site may exceed that of the blood glucose, since the specific activity of the latter will be falling with time. Such a region would therefore be likely to discharge CO_2 of relatively low specific activity early in an experiment but of relatively high specific activity later in the experiment. On the other hand a region with a small and/or rapidly mixing glucose pool would tend to discharge CO_2 of relatively high specific activity at an early stage.

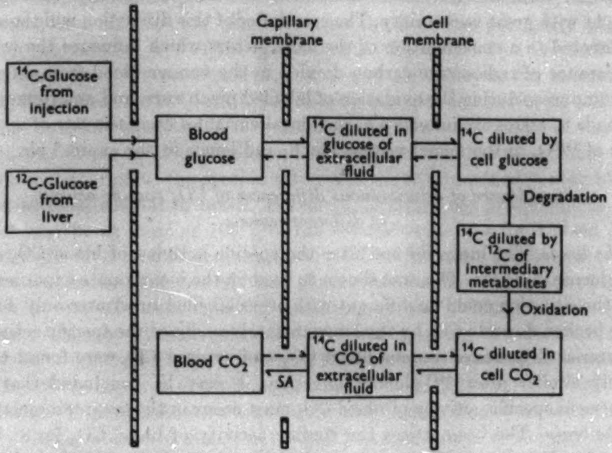
Capillary membrane
Cell membrane
^{14}C-Glucose from injection
^{12}C-Glucose from liver
Blood glucose
^{14}C diluted in glucose of extracellular fluid
^{14}C diluted by cell glucose
Degradation
^{14}C diluted by ^{12}C of intermediary metabolites
Oxidation
Blood CO_2
SA
^{14}C diluted in CO_2 of extracellular fluid
^{14}C diluted in cell CO_2

Fig. 9. Diagram illustrating stages in conversion of injected ^{14}C-glucose into exhaled $^{14}CO_2$. *SA* = *SA* of CO_2 of extracellular fluid.

It appears from the findings of Steele *et al.* (1956) that the concentration of free glucose in a tissue may give a fair indication of the size of its glucose pool, since the glucose pool of the body as a whole (defined as the glucose which readily dilutes injected ^{14}C-glucose) seems to consist almost entirely of free glucose dissolved in the blood and extracellular fluid. However, dilution of ^{14}C during its conversion from glucose to CO_2 might also result from mixing with pools of intermediary metabolites if these were present in any quantity, and evidence of such intermediate pools was found, in fact, by Sacks (1957). Omission of the effects of such pools from calculations of the proportion of CO_2 derived from glucose under various conditions may introduce appreciable inaccuracy. Examples of calculations which may be subject to such limitations are to be found in the report by Sacks (1956) concerning the origin of $^{14}CO_2$ in the effluent blood from brain and in the estimate by Baker, Shreeve, Shipley, Incefy & Miller (1954) of the proportion of expired $^{14}CO_2$ derived from glucose. The results of the latter authors are discussed in greater detail below (p. 506).

We had hoped when we began our experiments to be able to use arteriovenous differences in the specific activity of blood CO_2 in order to estimate the rate of oxidation of particular substrates in the individual regions studied. In fact, however, it has become clear that the influence of pool sizes and rates of mixing in various tissues so dominate the picture that estimates of rates of oxidation from arteriovenous differences in the radioactivity of CO_2 are fraught with great uncertainty. The remainder of this discussion will therefore be devoted to a consideration of the local factors which influence the rate of appearance of radioactive carbon dioxide in the venous blood from different vascular areas during the oxidation of labelled precursors, and an attempt will be made to assess the influence of such local variables upon the rate of appearance of $^{14}CO_2$ in the mixed venous blood, and hence in the expired air.

Significance of arteriovenous differences of CO_2 specific activity in different regions

The lungs. The lungs do not alter the specific activity of blood CO_2 after ^{14}C-glucose injection. This was shown to be so in the bicarbonate experiments, and the situation could be different with other labelled substrates only if they were broken down to CO_2 by the lungs themselves. Since the specific activities of arterial CO_2, mixed venous blood CO_2 and expired CO_2 were found to be closely similar after ^{14}C-glucose injection, it may be concluded that the changes in specific activity of blood CO_2 must occur in the greater circulation.

The brain. The brain raises the specific activity of blood CO_2 for at least 3 hr after ^{14}C-glucose injection in both dog and monkey. Qualitatively this agrees with what is known of cerebral metabolism. Brain appears to be dependent largely, if not entirely, on the blood sugar for its energy source (McIlwain, 1955), although Sacks (1956, 1958) found some evidence of utilization of fumarate and butyrate by human brain and Geiger (1958) also quotes some results suggesting non-glucose sources of substrates for brain metabolism. However, glucose remains by far the most effective antidote to hypoglycaemic coma and in man the brain consumes some 70% of the glucose entering the circulation from the hepatic veins and also some 24% of the oxygen intake of the body as a whole. It is therefore in accordance with expectation that our findings indicate a rapid conversion of blood glucose to CO_2 in the cerebral tissues. They also are in good agreement with the results of Sacks (1957), who made similar determinations of arteriovenous differences in specific activity of CO_2 across the brain following the administration of ^{14}C-glucose to man. The specific-activity-raising effect in the dog's brain was maintained for longer than in any other organ studied. This sustained output of $^{14}CO_2$ would be compatible with a small cerebral free-glucose pool in the presence of a high perfusion rate and a small bicarbonate pool; it could also be due in part to the oxidation in the later stages of an experiment of intermediates which had become labelled in earlier stages and were turning over relatively slowly. In connexion with the latter possibility it may be noted that J. R. Henderson & R. V. Coxon (unpublished) have found that the glycogen of brain attains a specific activity which greatly exceeds that of glycogen from liver or muscle some hours after an injection of ^{14}C-glucose.

Liver and splanchnic region. The situation here is complicated by the fact that some four-fifths of the blood entering the liver has previously passed through the vascular bed of the spleen or gut. However, if one regards the whole territory of the portal circulation as a single region so far as arteriovenous differences are concerned, its effect on the specific activity of CO_2 is a 'raising' one; and since the outflow from the hepatic veins constitutes about one quarter of the total venous return to the heart, the hepatic effluent will make a contribution to the specific activity of the CO_2 in mixed venous blood almost as great as that of brain. For there, although the arteriovenous difference may be as great as 100% of the arterial value, the total blood flow is about one fifth of that through the liver. The fact that the liver appears to be active in the oxidation of blood glucose in these experiments is of some interest in view of the difficulty of measuring glucose utilization in the liver in intact animals by conventional methods employing the arteriovenous difference of unlabelled sugar (cf. Myers, 1950) owing to the practically unique capacity of the organ to extrude sugar into the blood. The ability of liver cells to oxidize ^{14}C-glucose *in vitro* is of course well known from work with tissue slices (Bloom, Stetten & Stetten, 1953). The contribution of the gut to the $^{14}CO_2$ content of hepatic-vein blood is probably very small in fasting subjects, for Myers (1950) found only a very small glucose uptake by the gut in fasting men and the relatively high rate of utilization of glucose by isolated rat intestine reported by Fisher & Parsons (1953) was found in association with absorptive activity.

The limbs. For the limbs our findings were entirely at variance with those characteristic of the other regions so far discussed; for instead of a positive arteriovenous difference in the *SA* of the blood CO_2, there was for at least 2 hr after injection of labelled glucose a negative difference. This negative difference was considerably greater than could be accounted for by simple dilution of arterial $^{14}CO_2$ by $^{12}CO_2$ derived from oxidation of non-radioactive material in the limb, since the change in specific activity was several times greater than the arteriovenous differences in total CO_2. It could therefore only be due, like the similar negative difference found in experiments where ^{14}C-bicarbonate was injected (Coxon & Robinson, 1959), to dilution in the pool of CO_2 in the limb. Since about 40% of the cardiac output passes through tissues of the same general type as those making up the hind limb and since in the early stages of an experiment the lowering of specific activity of CO_2 as between samples from the femoral artery and femoral vein is about 50%, it is apparent that under these conditions about 20% (i.e. 50% of 40%) of the $^{14}CO_2$ of the blood would be drained off into such tissues each minute, the blood volume being approximately equal to the minute output of the heart. It also follows from the magnitude of the arteriovenous difference in specific activity across the hind limb—and across similar tissues such as those drained by the external jugular and lumbar veins—and from their total blood flow that some 40% of the blood going to make up the venous return to the right side of the heart will contain CO_2 with a specific activity less than that of arterial blood. In view of the magnitude of this *SA*-lowering effect the question arises as to whether any $^{14}CO_2$ is being produced from ^{14}C-glucose in the tissues of the limb during the time when the effect is evident, since any CO_2 so produced would be radioactive. The fact that the magnitude of the fall in *SA* of CO_2 across the limb in the presence of ^{14}C-glucose is of the same order as when ^{14}C-bicarbonate is injected (Coxon & Robinson, 1959) and no appreciable quantity of ^{14}C-glucose is at hand, may appear to support the suggestion that glucose is not metabolized in the resting limb. However, the conditions in the two cases are not truly comparable, for in the experiments with ^{14}C-glucose, the *SA* of the arterial blood CO_2 was rising owing to the metabolic activity of such organs as the brain, whereas in the experiments where ^{14}C-bicarbonate was injected, it was steadily falling. In experiments where bicarbonate was injected, isotopic equilibrium between the tissue and venous CO_2 was established in about 30 min, as is indicated by the cross-over of the arterial and venous specific activity–time curves; in the experiments where glucose was injected, on the other hand, the curves coincided only after 2–3 hr. As has been implied earlier in this discussion, the arteriovenous difference across a region, in the presence of changing specific activities of arterial CO_2 and of glucose, serves only to compare that region with others contributing to the $^{14}CO_2$ of the blood; and hence, in the case of the limb while the arteriovenous difference is negative, this may mean only that $^{14}CO_2$ production there is being overshadowed by its more rapid production elsewhere. There is other information from studies of arteriovenous differences for non-isotopic glucose (Andres, Cader & Zierler, 1956) which strongly suggests that glucose is not a major fuel of resting muscle in man, but our present results, though consistent with this suggestion, do not provide positive support for it in the dog. In addition to this factor, the sluggishness of mixing in the limb pool of CO_2 may be paralleled by a similar retarded mixing in the glucose pool, which again would lower the *SA* of $^{14}CO_2$ produced from it in the early stages of an experiment as compared with that evolved by oxidation in other organs when blood glucose was more immediately broken down.

Collateral evidence of the size and mixing rates of regional pools of glucose and carbon dioxide

In view of the inferences drawn in preceding paragraphs regarding the extent and the rates of mixing of the glucose and carbon dioxide pools of different organs, it is important to consider what evidence, if any, of an independent kind is available against which to check these inferences. Such evidence is fragmentary, but in Table 5 we have assembled values for the glucose and carbon dioxide content of several of the bodily regions which we have studied. Also, since we have given some prominence to blood flow as a possible limiting factor in the rate of mixing of tissue pools, the table also includes estimates from the literature of the blood flow through various regions. Some of the values in the table are from species other than the dog and are therefore only applicable to the present purpose in a very approximate way,

TABLE 5. **Estimation from published values of magnitude of organ pools of glucose and carbon dioxide and of their perfusion rates**

Organ ...	Brain	Liver	Kidney	Resting muscle	Bone	Authors
Proportion of cardiac output received (%)	4·9 (dog) 10·3 (monkey)	25·1	25·4	26	1·4	Levy & Blalock (1937)
Weight of organ (g/kg body-weight)	10 (dog) 23 (monkey)	35	6·6	450	170	Skelton (1927); Dhéré & Lapicque (1898); Schmidt, Kety & Pennes (1945); Green (1950)
Blood flow (ml./100 g organ)	54 (monkey)	80	400	6·4	0·67	Schmidt *et al.* (1945); Blalock & Mason (1936); Levy & Blalock (1937); Cargill & Hickam (1949); Himwich & Castle (1927) Green, Lewis, Nickerson & Heller (1944); Andres *et al.* (1956); Edholm, Howarth & McMichael (1945)
Glucose concentration in organ (mg/100 g);	8 (rat)	82	51	22	—	Gey (1956)
Relative blood-perfusion rate (ml./mg glucose in organ)	6·8	0·98	8·4	0·32	—	Robinson (1958)
Contribution to glucose pool of whole body (mg/kg body wt.)	0·8	29	3·3	99	—	Robinson (1958)
Total CO_2 concentration (m-mole/100 g tissue)	1·3 (cat, rabbit)	1·4	1·1	1·1	60	Irving, Ferguson & Plewes (1930); Drury, Wick & Almen (1956)
Relative blood-perfusion rate (ml./m-mole of organ CO_2)	42	57	390	6·4	0·01	Robinson (1958)
Contribution to body pool of CO_2 (m-mole/kg body wt.)	0·1	0·51	0·07	5·0	100	Robinson (1958)

but they probably give a fair indication of the broad differences between the regions in question.

Bearing these limitations in mind, it becomes apparent from the table that the brain and kidney have distinctly high blood-perfusion rates relative to their contents of glucose and might therefore be expected to be the sites of rapidly mixing glucose pools, as was deduced from the behaviour of the arteriovenous specific activity curves for CO_2. Liver and resting muscle have smaller blood flows in relation to their glucose contents, the ratio being lowest in the case of muscle; the latter value, therefore, is consistent with our findings, which indicated a comparatively slow release of $^{14}CO_2$ from the resting dog's leg in which muscle is a major constituent. In the case of liver there appears to be an inconsistency between the two types of evidence, but this may be connected with the unique capacity of the liver to release glucose into the blood and with its double circulation.

As regards the CO_2 pools of different regions, which have been discussed more exhaustively elsewhere (Coxon & Robinson, 1959), the general picture, based on estimates of CO_2 content and blood flow, conforms with what is inferred from the arteriovenous measurements. The position of the kidney in any classification based on pool size and blood flow is rendered uncertain by its particularly high blood flow (which makes arteriovenous differences harder to measure) and by the non-uniformity which must arise from the existence of different tubular segments containing urine of varying composition. Thus in a general way the information which is to hand on the pool sizes and blood-perfusion rates in the regions in which we have studied $^{14}CO_2$ release is compatible with the suggested interpretation of our findings.

Experiments with substrates other than glucose

When either acetate or palmitate instead of glucose was the source of $^{14}CO_2$, the arteriovenous differences in SA_{CO_2} were qualitatively similar to those found with glucose as the oxidizable precursor. However, the time course of the changes observed was different; in particular the peak in the arterial SA–time curve after the injection of ^{14}C-acetate appeared much sooner than in experiments with ^{14}C-glucose (cf. Fig. 8); and also the femoral $V:A$ ratio of specific activities reached unity considerably sooner than when glucose had been injected—though somewhat later than when, in other experiments, radioactive bicarbonate had been exogenously administered. These relationships would be compatible with the not unreasonable idea that the pool of acetate in the metabolizing tissues was much smaller than the glucose pool. The high initial $V:A$ ratio for the liver when labelled acetate was supplied, which exceeded that found with glucose as the source of labelling, suggests that the liver's pool of acetate is turning over more rapidly than its glucose pool. The fact that the $V:A$ ratio for specific activity reaches and exceeds unity in the hind limb more rapidly with ^{14}C-palmitate than with ^{14}C-acetate (Fig. 8) may possibly suggest some relation to Gordon's (1957) report on the extraction from peripheral blood of unesterified fatty acid, which may therefore be a favoured fuel for resting muscle. However, it should again be emphasized that the effects of local pools of precursor and product are such that the arteriovenous difference in specific activity will only provide an estimate of the rate of production of $^{14}CO_2$ in the region studied as compared with the rates of production in other regions, since these must determine the arterial concentration of $^{14}CO_2$ at any given time. The manner in which the various regions exert their influence on the time course of the specific activity of arterial CO_2 will be examined in more detail in the next section.

Effect of different regions on the time course of changes in specific activity (SA) of arterial carbon dioxide

The information discussed in the foregoing paragraphs provides a possible foundation for working out an empirical reconstruction of the arterial SA_{CO_2}–time curve by summing algebraically the SA-raising and SA-lowering effects of different regions. We did not actually measure blood flow, but the agreement between our measurements of cardiac output and those in the literature suggests that, as a first approximation, published figures may justifiably be used to compute the fractions of the cardiac output traversing the various regions, and thus their relative contributions to the total venous return. By combining such published figures for blood flow with our own figures for arteriovenous differences in SA_{CO_2}, it is possible to calculate, for the times at which our measurements were made, the total raising or lowering effect of each region studied upon the SA of the CO_2 in the mixed venous blood reaching the heart. This blood has only to pass through the lungs (where no detectable change in SA occurs) to become arterial blood, and hence changes with time in mixed venous SA will be reflected in rapidly succeeding changes in arterial SA_{CO_2}. Estimates of the raising and lowering effects of the organs studied by us are shown in the last line of Table 6. Now, since measurements of arteriovenous differences in specific activity represent for practical purposes instantaneous changes in specific activity of CO_2 in the blood circulating through the organs sampled, the algebraic sum of such changes should provide a measure of instantaneous change in mixed venous and hence in arterial SA_{CO_2}. These instantaneous changes are an index of the rate of change at that instant. Formally expressed this relationship may be written:

$$\alpha\left(\frac{dSav_1}{dt}\right)+\beta\left(\frac{dSav_2}{dt}\right)+\gamma\left(\frac{dSav_n}{dt}\right)=\frac{dSa_{Art}}{dt}, \tag{1}$$

where $dSav_1$, $dSav_2$, etc. are arteriovenous differences in SA_{CO_2} in regions

1, 2..., *n*, and α, β, ...γ are coefficients allowing for the fraction of the total venous return contributed by these regions.

In Fig. 10 arteriovenous differences in SA_{CO_2} in different organs are plotted against time. In Fig. 11 the algebraic sum of these arteriovenous differences is plotted against time, and, from the arguments advanced above, this curve should give a measure of the slope of the arterial SA_{CO_2} plotted against time. It will be seen that this latter curve must pass through a maximum and a minimum at the points in time where the horizontal axis is cut in Fig. 11. Once the changes in slope with time are established, a theoretical arterial SA_{CO_2}-time curve can be constructed, and, by choosing a suitable scale, can be superimposed upon an observed curve. This has been done in Fig. 12, and on comparing the experimental with the calculated graph it will be apparent

TABLE 6. Effects of positive and negative arteriovenous differences in specific activity of blood carbon dioxide (SA_{CO_2}) in various organs on SA_{CO_2} of mixed venous blood returning to heart

Arteriovenous increment or decrement in SA_{CO_2}	Time (min)					
	10	25	40	60	80	100
In liver	+1·2	+2·0	+2·3	+1·0	+0·6	+0·5
In kidney	+1·7	+1·4	+0·5	0	0	0
In brain	+2·0	+2·0	+2·0	+1·9	+1·7	+1·5
In limbs	−2·5	−3·5	−4·4	−3·8	−1·7	—
Over-all change in SA_{CO_2} during one circulation	+2·4	+1·9	+0·4	−0·9	+0·6	—

The arteriovenous increments and decrements tabulated are derived from the measured values in a series of experiments chosen so that the peak in the arterial specific activity–time curve lay near 50 min. The observed arteriovenous differences were multiplied by a factor such that the peak arterial SA_{CO_2} would correspond to 15 counts/min/μmole, and in the case of the brain were divided by 5 since the blood flow through the brain is one fifth of that through the other organs listed.

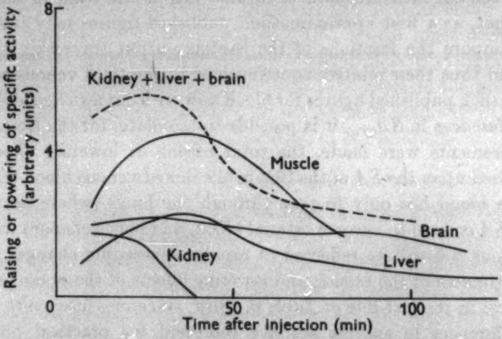

Fig. 10. Calculated effects of venous outflow from brain, liver and kidney in raising, and of resting muscles in lowering, the specific activity of carbon dioxide in mixed venous blood.

that there is very good correspondence up to 40 min, which is shortly before the *SA* reaches its peak at $t_{\text{max.}}$; beyond this point agreement is less good. Since the predicted curve was based, owing to scarcity of values, on somewhat imprecise calculations, some doubt must remain as to whether the relatively slight deviations between it and the experimental curve are real. If, without prejudging this question, a cause of the deviation is sought within our admittedly incomplete data, it will be found that the principal reason for the

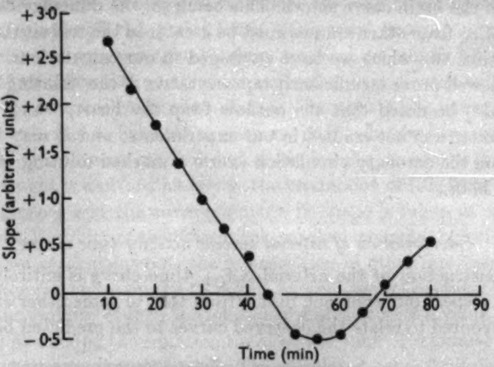

Fig. 11. Change of slope with time of arterial SA_{CO_2}–time curve predicted from results in Table 6.

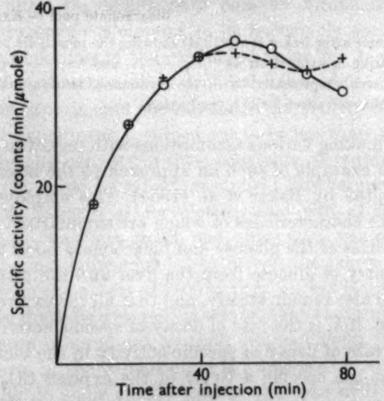

Fig. 12. Comparison of shape of an experimental curve (○) of arterial SA_{CO_2} against time with that of a curve predicted from Fig. 11 (+). The two curves have been drawn to coincide both as regards slope and height at the 10-min point; the theoretical curve at the other points shown has been assigned a slope according to the relationship with time depicted in Fig. 11.

secondary rise in the predicted curve between 70 and 80 min lies in the continued discharge of CO_2 of relatively high *SA* from the brain. This is evident from inspection of Fig. 10, but it must be emphasized that the brain curve in that figure was based on only a single experiment. This was the only experiment of the kind performed on a dog, but our other experiments in which venous blood from the brains of monkeys was sampled, and also the results of Sacks (1957) on the human brain, tend to confirm in a qualitative way the accuracy of the brain curve shown. This being so, the diluting effect of blood of lower SA_{CO_2} from other sources must be greater in the real situation than in the theoretical one which we have envisaged in our calculations. Hind-limb results may well prove insufficiently representative of the 'diluting' organs and it should also be noted that the outflow from the heart, which has a high metabolic rate, was not studied in our experiments; and it may be that the effluent from the coronary circulation exerts a marked diluting effect during the second hour.

Interpretation of arterial specific activity–time curves

Our reconstruction of the arterial SA_{CO_2}–time curve is entirely empirical and in this respect our approach differs from that of some other authors who have endeavoured to relate the observed curves to the predicted behaviour of model systems by making various assumptions with regard to the behaviour of these systems. An example of such an approach to the interpretation of the curve is that adopted by Baker *et al.* (1954). This employs a hypothetical two-pool model, the characteristics of which are summarized in Fig. 13. It is assumed that the sizes of the glucose and bicarbonate pools are constant and that the rate of entry of glucose from the liver and the rate of discharge of CO_2 from the lungs also remain steady, and that all the expired CO_2 is derived from glucose. Then, if λ_1 is the rate of decay of specific activity in the glucose pool and λ_2 is the rate of decay of specific activity in the bicarbonate pool, it can be shown that the specific activity of the expired CO_2 (b) at time t is given by:

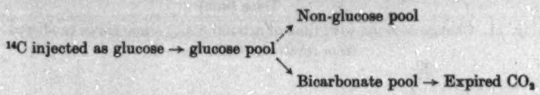
Non-glucose pool
^{14}C injected as glucose → glucose pool
Bicarbonate pool → Expired CO_2

Fig. 13. Simplified scheme after Baker *et al.* (1954) showing the metabolic path of ^{14}C injected as glucose and appearing in expired air as $^{14}CO_2$. ——— and ··········· represent events in the real animal. Only events represented by ——— are assumed to occur in deriving a theoretical '100% CO_2 from glucose' curve for CO_2 production.

$$b = a_0 \frac{\lambda_2}{\lambda_2 - \lambda_1} \left(e^{-\lambda_1 t} - e^{-\lambda_2 t}\right), \tag{2}$$

where a_0 is the initial specific activity of the body's glucose.

This expression is valid so long as the injected ^{14}C-glucose mixes rapidly with the body's pool of ^{12}C-glucose; it further requires that there be a relatively rapid and irreversible transformation of glucose to carbon dioxide without serious hold-up in intermediate pools, and that the CO_2 pool itself be effectively homogeneous. It is also clear that algebraically the equation is only meaningful so long as λ_1 and λ_2 are not equal to each other, since, if they were, the denominator of the right-hand side would become zero.

Using such a model, Baker *et al.* calculate the rate of change of the *SA* of expired CO_2 and call this their '100% CO_2 curve', recognizing that it will differ from the real curve obtainable from a resting animal in which the R.Q. will be less than unity and much of the expired carbon dioxide will in reality be derived from sources other than glucose. The '100% CO_2 curve' so calculated turns out in fact to be of the same shape as the observed curves from four human subjects who were studied, but is displaced upwards on the *SA* axis. This displacement is ascribed mainly to the production of CO_2 from precursors other than glucose and the correspondence in shape is taken as evidence of the general applicability of the theoretical two-pool model to the living subject; since, as Baker *et al.* point out, the interposition of other intermediate pools would displace the curve on the time axis and change its contour. In further support of the validity of their model, Baker *et al.* calculate a value for λ_2 from equation (2) by solving the equation for λ_2 at the time of maximal *SA* ($t_{\text{max.}}$) taken from their experimental curve. The calculated value for λ_2 is then compared with a value for λ_2 directly determined on other experimental subjects, obtained by labelling the bicarbonate pool by means of an injection of $NaH^{14}CO_3$. The agreement is claimed to be reasonably good, but, in fact, in one of their four experiments the calculated value for λ_1 was nearer to the observed value of λ_2 than was the calculated value of λ_2. Secondly, since in three of their four experiments the calculated value of λ_2 was greater than λ_1, while in the other experiment λ_2 turned out to be less than λ_1, it may be questioned whether the non-equality of these constants, as required in the application of equation (2), could be safely presumed. Furthermore, the number of points available to Baker *et al.* for plotting that part of their 100% curve which preceded $t_{\text{max.}}$ was very small, and, since $t_{\text{max.}}$ was not represented by a really sharp inflexion in their published curves, the exactness of correspondence in contour with the observed '100% CO_2' curves is difficult to establish unequivocally. It must be granted, however, that the general impression of good correspondence is borne out by their graphs. Apart from these possible inherent weaknesses in the validation of the two-pool model by Baker *et al.*, our present results do show clearly that at the time of the peak in arterial SA_{CO_2} the bicarbonate pool in our dogs was not homogeneous.

We have made no measurements of the radioactivity of blood glucose but it is evident from the results of Steele *et al.* (1956) that mixing in the glucose pool of a dog was incomplete until about 60 min from the time of a single intravenous injection of ^{14}C-glucose. This is a little after $t_{\max.}$ on the arterial SA–time curve in many of our experiments. Now Baker *et al.* have made calculations of the proportion of expired CO_2 coming from the oxidation of glucose by assuming that the fraction of expired CO_2 derived from glucose will have at $t_{\max.}$ the same SA as that of the body's glucose pool at that time, the glucose pool being sampled from plasma glucose. This, in view of the probably incomplete mixing of the glucose pool and the certainly incomplete mixing of the CO_2 pool at this time, is clearly not strictly accurate. For example, CO_2 derived from oxidation of ^{14}C-glucose in the brain at $t_{\max.}$ will still be undergoing dilution in the CO_2 pools of the limbs, as is illustrated in Fig. 10.

Our observations point to the desirability of applying theoretical arguments in terms of pools and turnover rates to regions of the body rather than to the whole body. Ideally the regions studied would be narrowed down to the point where they comprised morphologically and metabolically homogeneous cell populations with a uniform and constant vascular supply, but in practice the study of quite crudely demarcated subdivisions of the body may be expected to give a much truer picture of the course of events than is attainable by treating the whole body as a single catenary system. This has been appreciated by a number of authors, as is evident from Robertson's (1957) review of models employed in the interpretation of isotopic data from intact animals. However, comparatively few measurements have been made on subcompartments of the animal body, and the present results appear to suggest that such studies may go a considerable way towards bridging gaps between physiological events in the real animal and their counterparts in mathematically idealized model systems.

It should be noted in conclusion that, when experiments are designed with the primary aim of determining the rate of oxidation of a labelled substrate in an intact animal, the complexities of the problem can be considerably reduced by the employment of a continuous infusion instead of an instantaneous injection of the isotopic metabolite, so that its specific activity in the arterial blood may be kept reasonably constant. This technique has been successfully used by Steele *et al.* (1956) and by Searle, Strisower & Chaikoff (1956).

SUMMARY

1. Measurements of the specific activity of carbon dioxide in arterial blood and in the venous blood from a number of organs have been made after the injection of ^{14}C-glucose, ^{14}C-acetate and ^{14}C-palmitate into dogs and monkeys.

2. Organs can be classified into those which, during the hour following the injection, either raise or lower the specific activity of the carbon dioxide in blood passing through them.

3. Arteriovenous differences across brain, liver and kidney showed a raising of specific activity, resting muscles a lowering.

4. On the basis of the different behaviour of different organs in respect of $^{14}CO_2$ production from ^{14}C-glucose an empirical explanation is offered of the time course of the changes in specific activity of expired CO_2 during the oxidation of ^{14}C-glucose.

5. The application of these findings to model systems used in the interpretation of data from animal experiments employing isotopic tracers is discussed.

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SOME PERIPHERAL ACTIONS OF DIPHTHERIA TOXIN

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There have been many attempts to ascertain the mechanism of the circulatory failure in diphtheria intoxication. None of them have been entirely satisfactory. Some workers, Romberg, Pässler, Brühns & Müller (1899), and Yabe (1922) thought it was due to the effect of toxin on the vasomotor centre, whereas others, Brodie (1899), Harding (1920), Myers (1933), and Friedemann & Elkeles (1934) considered that it was due to a direct effect on the peripheral blood vessels. Porter & Pratt (1914) found no evidence for malfunction of the vasomotor centre in diphtheria intoxication, and MacCallum (1914) reported that the toxin had no effect on the working capacity of the heart. In a few experiments in cats we have determined that the circulatory failure does not occur until the terminal stages of intoxication, and that when it does occur, death intervenes very shortly afterwards. The interval is so short that direct observations on the effects of the toxin on the circulatory system are not practicable. It probably also accounts for the conflicting views of the earlier workers. Agarwal & Holt (1959) analysed the positive Schick reaction obtained by injecting diphtheria toxin intradermally into guinea-pigs and showed that the skin vessels become refractory to adrenaline and noradrenaline in the Schick areas. They thought that the toxin may be acting on the smooth-muscle cells of the arterioles and capillaries. Hence they tested the effects of diphtheria toxin on another plain muscle, namely rabbit iris. The response of this muscle to doses of adrenaline was also depressed.

The present study was undertaken to get a better understanding of the effects of diphtheria toxin on other plain muscles such as the nictitating membrane of the cat and the uterus of the rabbit. In an attempt to ascertain the mechanism by which diphtheria toxin decreases the excitability of muscles, we studied its effect on the loss of potassium from isolated guinea-pig auricles and from the rabbit uterus. We also investigated the differences in the electrolyte content of various tissues in normal and diphtheria-intoxicated guinea-pigs.

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METHODS

Toxins. The toxin was prepared as described by Agarwal & Holt (1959). It was stabilized with tryptone and stored as a freeze-dried powder in a vacuum desiccator. Throughout the text 'MLD' refers to the minimal lethal dose in guinea-pigs: antitoxin is M.R.C. standard.

Experiments on the cat's nictitating membrane. Cats weighing 2–3 kg were anaesthetized with ether and doses of toxin varying from 0·05 to 4 MLD were injected into the left nictitating membrane in a constant volume of 0·01 ml. saline. The same quantity of boiled toxin was injected into the right nictitating membrane to serve as a control. Later, under chloralose anaesthesia, the contractions of the nictitating membranes on both sides in response to stimulation of the cervical sympathetic nerves and to the injection of drugs were recorded with isotonic levers having frontal-writing points. The levers were carefully balanced so that the magnification (×10) and the tension were the same on each. The cervical sympathetic nerves were stimulated preganglionically, with a Palmer student stimulator giving square wave pulses of 0·35 msec duration at a rate of 30 c/s. The strength of the stimuli was varied as required. Drugs were either injected intravenously via a cannula in the femoral vein, or into the central end of the lingual artery. In the latter case, the internal carotid artery was occluded while injections were being made. In some experiments the effects of active and passive immunization were observed. For active immunization four injections of 0·5 ml. purified alum-precipitated diphtheria toxoid (P.T.A.P.) were made into the axillae and groins of the cat. Three weeks later two doses of 0·5 ml. of formol-toxoid (30 Lf/ml.) were injected subcutaneously. Within 10–12 days a good antibody response developed. For passive immunization the cats received 100 u. of diphtheria antitoxin intravenously 6 hr before the injection of toxin.

Experiments on the rabbit uterus. For these experiments rabbits were anaesthetized with pentobarbitone sodium (30 mg/kg). The abdomen was opened and the uterine horns exposed. Doses of toxin varying from 0·1 to 0·4 MLD were injected in 0·01 ml. saline with a micrometer syringe into the subserous coat of the left uterine horn, and the same amount of boiled toxin into the right uterine horn, the latter serving as a control. Forty-eight hours later, the rabbits were anaesthetized with urethane (1·8 g/kg) subcutaneously, and the abdomen was re-opened. The caudal ends of the uterine horns were clamped and firmly fixed. The contractions of approximately equal lengths of the two horns were then recorded with frontal-writing levers which were carefully balanced so that the magnification (×10) and the tension on both sides were the same. Since it was not possible to obtain exactly equal lengths of both left and right horns to make the records, it was not permissible to compare the absolute contractions. Therefore the dose-response curves to adrenaline injected into the femoral vein were constructed for each horn and the slopes of the curves were compared.

K^+ efflux from isolated guinea-pig auricles. Guinea-pigs weighing 300–600 g were stunned by a blow on the head and bled from the carotid arteries. The thorax was immediately opened and the heart dissected out and placed in Krebs–Henseleit solution containing NaH_2PO_4 instead of KH_2PO_4. The solution had the following composition (%): NaCl 0·69, KCl 0·035, $CaCl_2$ 0·0298, $MgSO_4$, $7H_2O$ 0·029, $NaHCO_3$ 0·21, NaH_2PO_4 0·016, glucose 0·2 and distilled water to 100, and is referred to as Krebs's solution in the text. The heart was gently squeezed to remove blood and transferred to a Petri dish containing oxygenated Krebs's solution. The auricles were dissected free from the ventricles and one end was attached to a glass hook in an 8 ml. bath and the other to a heart lever. For estimation of the potassium efflux a technique similar to that of Holland (1957) was employed. The normally beating auricles were allowed to equilibrate in Krebs's solution for 30 min. At the end of this period they were washed four times with a potassium-free Krebs's solution. After the washing procedure, 4 ml. potassium-free solution was introduced into the bath. After varying intervals of time the solution was aspirated from the bath and its potassium content was estimated, using a Gallenkamp flame photometer. After each period in K-free Krebs's solution the potassium content of the auricles was restored by immersing them in the solution containing twice the usual content of potassium chloride for 10 min.

Estimation of K^+ efflux and the K^+ content of isolated rabbit uterine horns. Female non-pregnant rabbits were lightly anaesthetized with pentobarbitone sodium (30 mg/kg) and ether. The uterine horns were exposed. Two-fifths MLD diphtheria toxin contained in 0·01 ml. NaCl 0·85% (w/v) was injected into the left uterine horn with a micrometer syringe. The right uterine horn served as a control and was injected with the same amount of boiled toxin. Forty-eight hours later the rabbits were stunned by a blow on the head and bled from the carotid arteries. The two uterine horns were removed and placed in a Petri dish containing Tyrode solution of the following composition (%): NaCl 0·9, KCl 0·042, $CaCl_2$ 0·024, $MgSO_4$ 0·0075, $NaHCO_3$ 0·05, glucose 0·1, distilled water to 100. Approximately equal lengths of the two horns were taken and mounted in a 6 ml. bath. The uterine horns were allowed to equilibrate for 30 min in the Tyrode solution, the temperature of the bath being maintained at 26° C throughout the experiment. After the 30 min period of equilibration the two baths were emptied and the uterine horns washed four times with K^+-free Tyrode solution (KCl omitted). They were then allowed to contract for 5 min in 5 ml. K^+-free Tyrode solution. The solutions were then aspirated, and the procedure was repeated eight times. The samples were pooled and their potassium content estimated. This procedure ensured that a maximal outward gradient for potassium was maintained throughout the experiment.

Estimation of sodium and potassium. The potassium in the bath fluid from the experiments with isolated guinea-pig auricles and isolated rabbit uteri was estimated with a flame photometer. The same method was also employed to determine the sodium and potassium content of tissues from normal and intoxicated guinea-pigs. For this purpose the tissues were blotted and weighed (wet weight) before drying to a constant weight. They were then digested in 0·5 ml. of HNO_3 (Analar) diluted with 4·5 ml. of glass-distilled water and their sodium and potassium contents estimated by the same procedure.

RESULTS

The response of the cat's nictitating membrane

In preliminary experiments varying quantities of diphtheria toxin were locally injected into the nictitating membrane of several cats to find a dose that would not produce any visible inflammatory reaction. With a dose of 4 MLD toxin the injected eye became congested and there was excessive watering and photophobia. The nictitating membrane was oedematous and hyperaemic. With doses of less than 1 MLD there was no visible reaction. Hence in all the experiments 0·4 MLD or less was employed.

Figure 1 shows the results of preganglionic stimulation of the cervical sympathetic chain and of intravenous administration of adrenaline or noradrenaline 48 hr after the injection of 0·1 MLD diphtheria toxin into the left nictitating membrane. A preganglionic stimulus of 0·7 V produced a contraction of the control membrane but elicited no response from the toxin-treated membrane. When the strength of the stimulus was increased to 2 V both membranes contracted but the response of the toxin-treated membrane was less than that of the control. The intravenous injection of 15 μg adrenaline caused both membranes to contract but the response of the toxin-treated membrane was less than that of the control. The responses in the two membranes to intravenous injection of 15 μg noradrenaline were approximately equal.

Ten minutes after the subcutaneous injection of 10 mg cocaine hydrochloride the responses of the nictitating membranes to preganglionic stimulation of the cervical sympathetic nerves and to intravenous administration of adrenaline and noradrenaline were potentiated in the control and in the toxin-treated nictitating membranes, but the potentiation was less in the toxin-treated membrane than in the control. This is illustrated in Fig. 1.

Forty-eight hours after the injection of 0·05 MLD toxin, 15 μg of adrenaline injected into the femoral vein caused almost equal contractions of the toxin-injected and the control membranes. When, however, 5 μg adrenaline hydrochloride solution was injected into the central end of the lingual artery on the appropriate side, the toxin-treated membrane contracted less than the control. The responses to acetylcholine, potassium chloride and barium chloride similarly injected were less on toxin-treated membranes. This is illustrated in Fig. 2.

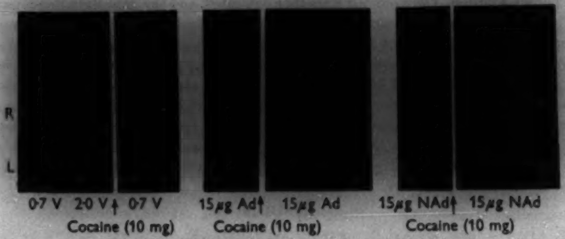

Fig. 1. The effect of a local injection of diphtheria toxin on the responses of the nictitating membrane of a cat anaesthetized with chloralose (80 mg/kg). 0·1 MLD toxin was injected into the left nictitating membrane and the same amount of boiled toxin into the right nictitating membrane 48 hr previously. Ad, adrenaline; NAd, noradrenaline. 0·7 V and 2·0 V, strength of preganglionic stimulation.

Experiments in three cats showed that after the injection of 0·05 MLD toxin into the nictitating membrane the reduction in its ability to contract to intravenous doses of adrenaline and to stimulation of the cervical sympathetic nerve was manifest as early as 18 hr and was present after 17 and 27 days.

Effects of passive immunization, active immunization and injection of toxin–antitoxin mixtures. The following experiments illustrate the specificity of the action of diphtheria toxin. (1) Forty-eight hours after the injection of 1/5 MLD diphtheria toxin into the nictitating membrane of a passively immunized cat, the responses to preganglionic stimulation and to intravenous doses of adrenaline were not depressed, as is illustrated in the experiment Fig. 3. (2) In one experiment 1/7 MLD diphtheria toxin mixed *in vitro* with 0·06 u. of diphtheria antitoxin was injected into the left nictitating membrane, and the same amount of boiled toxin mixed with 0·06 u. of diphtheria antitoxin into the right nictitating membrane to serve as a control. Forty-eight hours later the responses of the two nictitating membranes to the intravenous or intra-arterial injections of adrenaline or noradrenaline, or to the intra-arterial injections of acetylcholine and KCl, were the same. (3) Three cats were actively immunized with P.T.A.P. and formol toxoid. 0·05 MLD diphtheria toxin was injected into the left nictitating membrane and the same quantity of boiled toxin into the right nictitating membrane. Forty-eight hours later the responses of the two nictitating membranes to preganglionic

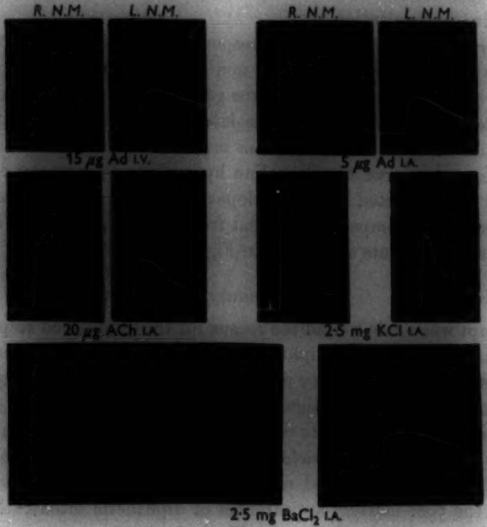

Fig. 2. The effect of locally injected diphtheria toxin on the responses of the nictitating membranes of a cat anaesthetized with chloralose (80 mg/kg). 0·05 MLD of boiled toxin injected into the right, and of unboiled toxin into the left, nictitating membranes 48 hr previously. *R.N.M.*, right nictitating membrane; *L.N.M.*, left nictitating membrane; Ad, adrenaline; ACh, acetylcholine; I.V., via femoral vein; I.A., via central end of the lingual artery.

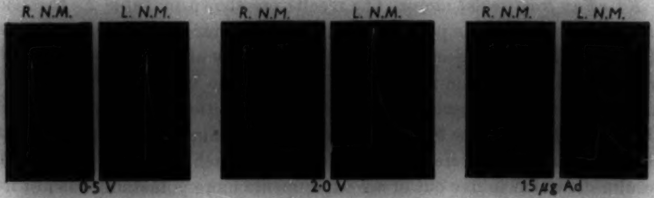

Fig. 3. Effect of passive immunization on the depressed responses of the cat's nictitating membrane produced by local injection of diphtheria toxin. 100 units of diphtheria antitoxin were given intravenously 6 hr before the injection of 1/5 MLD toxin into the left, and the same amount of boiled toxin into the right nictitating membrane. Records taken in chloralose anaesthesia (80 mg/kg) 48 hr after injection of toxin. Conventions as for Figs. 1, 2.

sympathetic stimulation, or injections of adrenaline or noradrenaline, were found to be the same.

These results show that the reduced responses of the cat's nictitating membrane after local injections of diphtheria toxin can be specifically neutralized by the previous administration of diphtheria antitoxin locally or systemically, and by active immunization.

Effect on rabbit uterine horns. Dose-response curves of the effect of adrenaline on the two uterine horns in normal non-pregnant rabbits were determined both *in vitro* and *in vivo*. The two horns gave parallel dose-response curves in experiments *in vivo*, but not in the isolated organ bath. Hence the effect of local injection of toxin was studied *in vivo*. In three experiments in which the dose-response curve to adrenaline hydrochloride was compared in the normal and toxin-injected horns, the slope of the curve from the toxin-injected horn was depressed compared with that from the control horn. The results of one of these experiments are shown in Fig. 4.

Potassium efflux

An attempt was made to find the reason for the diminished responses of the smooth muscle produced by diphtheria toxin. It was possible that the toxin interfered with the permeability of the cell membrane and consequently with the excitatory wave. To investigate this possibility the following experiments on the K^+ efflux and electrolyte control of tissues were carried out.

K^+ efflux from guinea-pig auricles. Guinea-pigs were injected subcutaneously with 30 MLD toxin and killed after 24 hr, when they were moribund. In every case the post-mortem appearances of diphtheria intoxication, notably adrenal haemorrhages, were present. The leakage of potassium into a potassium-free Krebs's solution from the auricles of these intoxicated animals and from the auricles of normal animals was measured. The results given in Table 1 show that diphtheria intoxication produces no significant change in the permeability of the auricles to potassium.

K^+ efflux from rabbit uterus. The results are shown in Table 2. In each of the six experiments the potassium efflux was greater from the control muscle than from the toxin-injected muscle. Immediately after the 40-min period of immersion in potassium-free Tyrode solution, the uteri were immersed for four successive 5 min periods in K-free Tyrode solution containing adrenaline. During the first of these 5 min periods, 1 μg adrenaline was added to the bath; the dose was then increased by 1 μg during each successive period. The results show that in five out of six experiments more potassium was lost from the control muscle than from the diphtheria-toxin-injected muscle.

Electrolyte content of guinea-pig tissues. Guinea-pigs were given a subcutaneous injection of 35 MLD diphtheria toxin and the sodium and potassium contents of various tissues were determined 24 hr later. The results obtained were compared with those from normal animals. The results given in Table 3 show that in the intoxicated animal there was a considerable and significant increase in the sodium and potassium contents of the thoracic and abdominal aorta, while the other tissues were not significantly affected.

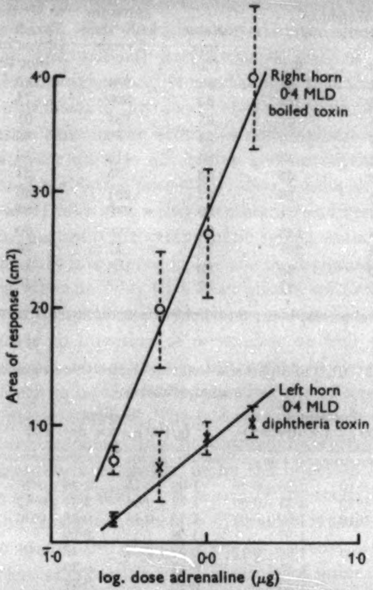

Fig. 4. Effect of locally injected diphtheria toxin on the dose-response curve to adrenaline on the rabbit uterus *in vivo* obtained in urethane anaesthesia (1·8 g/kg subcutaneously). 48 hr previously 0·4 MLD of boiled toxin injected into the right, and unboiled toxin into the left uterine horn. The area of each response was measured with a planimeter in order to include the duration as well as the linear height of the responses; the dotted lines give the range of responses obtained; semi-log. scale.

Table 1. Mean potassium efflux from isolated auricles of 5 normal and 5 diphtheria-intoxicated guinea-pigs beating in K⁺-free Krebs's solution

Time (min)	5	10	15	20	25	30	Total leakage (mg/100 g)
	Potassium leakage (mg/100 g)						
Animals							
Normal							
A	18	27	35	40	45	45	210
B	94	141	186	207	237	240	1005
Intoxicated							
A	13	24	31	40	36	45	189
B	75	133	183	222	201	263	1038

A = K⁺ mg/100 g wet wt., *B* = K⁺ mg/100 g dry wt.

TABLE 2. Potassium efflux from normal and toxin-injected rabbit uterine horns during contraction in K^+-free Tyrode solution

Expt. no.	Tissue	Weight of uterus (mg)		Water loss (%)	K^+ loss 8 × 5 min exposure to K^+-free Tyrode	K^+ loss 4 × 5 min exposure to K^+-free Tyrode + adrenaline	K^+ total loss during expt.	K^+ in tissue at end of expt
		wet	dry			(mg/100 g dry wt.)		
1	*A*	233	34	85	484	115	600	1147
	B	163	28	82	363	74	437	1344
2	*A*	345	51	85	134	91	225	655
	B	374	59	84	86	82	168	1338
3	*A*	341	53	84	117	84	201	1200
	B	399	65	83	95	21	106	1500
4	*A*	208	26	87	291	140	431	1926
	B	197	21	89	249	185	434	1932
5	*A*	759	143	81	120	60	180	983
	B	919	257	72	47	33	80	1182
6	*A*	376	66	83	105	53	158	841
	B	486	87	82	92	46	138	931

A = normal uterine horn; *B* = diphtheria-toxin-injected uterine horn.

TABLE 3. The effect of 35 MLD of diphtheria toxin on electrolytes in guinea-pig tissues 24 hr after its administration

	Potassium (g/100 g ± S.E. dry wt.)		Sodium (g/100 g ± S.E. dry wt.)		Water loss (%)		No. of animals	
Tissue	*C*	*T*	*C*	*T*	*C*	*T*	*C*	*T*
Thoracic aorta	1·27 ± 0·10	2·25 ± 0·26	1·08 ± 0·15	2·26 ± 0·25	70·7	83·9	8	8
Abdominal aorta	1·36 ± 0·32	3·31 ± 0·60	1·79 ± 0·44	3·34 ± 0·62	40·4	65·7	8	8
Uterus	1·76 ± 0·16	1·89 ± 0·07	1·10 ± 0·11	0·98 ± 0·06	83·5	84·7	8	8
Auricle	2·01	1·97	1·06	0·99	79·8	82·7	4	4
Ventricle	1·79 ± 0·05	1·66 ± 0·12	0·52 ± 0·07	0·43 ± 0·01	80·0	79·4	7	8
Hypothalamus	1·55	1·55	0·43	0·39	77·9	76·4	3	4
Spinal cord	1·03	1·23	0·40	0·47	70·4	71·0	3	4

C = control animals, *T* = toxin-treated animals.

DISCUSSION

The mechanism of the circulatory failure in diphtheria intoxication has remained a controversial problem ever since the discovery of diphtheria toxin. From the beginning the attention of the clinicians and pathologists was directed towards myocardial failure as the chief cause of death in diphtheria, because most of the patients dying from it showed parenchymatous and fatty degeneration of the cardiac muscle. MacCallum (1914), however, showed that myocardial weakness is not primarily responsible for death in diphtheria intoxication. The other possibility, namely the paralysis of the vasomotor centre leading to circulatory failure, has been discussed and rejected by Friedemann & Elkeles (1934) and by the experimental work of Porter & Pratt (1914). Furthermore, one of us (S.C.A.) has observed that when guinea-pigs, rats and mice are injected with diphtheria toxin intracerebrally, they do not die from circulatory failure but develop an ascending diphtheritic polyneuritis. If the vasomotor centre is affected by diphtheria toxin it might surely be expected to produce circulatory failure in these animals. Friedemann & Elkeles (1943) felt that the damage to the blood vessels is the most likely cause of the circulatory collapse. This view is supported by the general vasodilatation, capillary stagnation and adrenal haemorrhages seen in different animals dying from diphtheria intoxication. It was thought that the toxin may either damage the vasomotor fibres in the blood-vessel wall or diminish the reactivity of the smooth muscle cells in the arterioles and capillaries to the naturally liberated vasoconstrictor amines, adrenaline and noradrenaline. Agarwal & Holt (1959) suggested the latter possibility. Our results show that the toxin reduces the responses of cat's nictitating membrane to adrenaline or noradrenaline either liberated naturally at sympathetic nerve endings, as during preganglionic stimulation, or introduced from outside. A similar effect is produced in the rabbit uterus. It is unlikely that these effects are produced by a reduction in the amount of sympathomimetic amines released at the nerve endings, because the responses to injection of adrenaline as well as those to nerve stimulation are reduced. The increased destruction of the sympathomimetic amines is also unlikely to be the explanation, as the effects of acetylcholine, KCl and $BaCl_2$ are also diminished. The fact that the effect of the toxin on the nictitating membrane responses can be blocked by active and passive immunization with diphtheria antitoxin, or by the injection of toxin-antitoxin mixtures, suggests that the inhibition normally produced is specific for the toxin. The reduction in the contractility of smooth muscle could be effected by a defect in the movement of potassium ions across the cell membrane. The studies on the loss of potassium from the isolated guinea-pig auricles from normal and diphtheritic guinea-pigs do not show any differences in the potassium loss. It may be that the toxin given parenterally does not affect the permeability of the auricles. The finding that in diphtheria-intoxicated guinea-pigs the potassium content of the thoracic and abdominal aortic wall is considerably higher than in normal animals, while in other tissues including the uterus and the auricles it is not affected, suggests that the vascular system is much more sensitive to the action of the toxin. Hence it appears that the toxin affects the permeability of the cell membrane to potassium ions in some tissues but not in all. While it has been shown that toxin increases the sodium and potassium content of various tissues and that it inhibits the loss of potassium from smooth muscle cells, it is not possible to say whether these changes are the cause or the effect of the decreased muscular movement produced by local injections of diphtheria toxin. It might be that the increase in potassium content of the cell is the result of disturbances in the glycolytic cycle of the cell.

SUMMARY

1. The local injection of 0·05 MLD diphtheria toxin into the cat's nictitating membrane depressed its responses to preganglionic stimulation, intravenous injection of adrenaline or noradrenaline and to intra-arterially (lingual arteries) injected acetylcholine, KCl and $BaCl_2$.

2. The potentiation of the responses of the nictitating membrane to sympathomimetic amines, produced by cocaine, was reduced by the local injection of diphtheria toxin.

3. The action of the toxin on the smooth muscle of nictitating membrane was blocked by active or passive immunization. The local injection of toxin-antitoxin mixture produced no inhibition of membrane response.

4. The response of the rabbit uterus to adrenaline was reduced by the local injection of diphtheria toxin.

5. The efflux of potassium from isolated guinea-pig auricles was not affected by the systemic administration of diphtheria toxin, but the local injection of the toxin into the rabbit uterine horns inhibited the leakage of potassium into potassium-free Tyrode solution.

6. The potassium and sodium contents of the abdominal and thoracic aortic wall in diphtheria-intoxicated guinea-pigs were increased.

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THE DEPENDENCE OF TENSION UPON EXTENSION IN THE STRETCH REFLEX OF THE SOLEUS MUSCLE OF THE DECEREBRATE CAT

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In their description of the stretch reflex, Liddell & Sherrington (1924) stated that 'so long as the stretch increases, the reflex continues to increase'. The importance of this finding for an understanding of posture has long been recognized; but their statement has an additional interest in that it relates the magnitude of a reflex response to the size of the physiological stimulus which elicited it. The present paper describes a more detailed study of this relation between stimulus (muscle extension) and its reflex response (muscle contraction) in the hope that increased knowledge of the response of the reflex arc will contribute to an understanding of its detailed mechanisms.

The relation between tension and extension of the stretch reflex has been previously studied by applying a series of extensions of different lengths to the muscle (Koella, Nakao, Evans & Wada, 1956; Granit, 1958). In the present study the stretch reflex has been elicited by extending the muscle at a slow constant velocity, while recording both its tension and the extension applied to it. In addition, the present paper describes some experiments on the effect of extending the soleus muscle upon the tension developed in it on stimulation of its motor nerve. These experiments show that the 'tetanic fusion frequency' of soleus may vary with its length—a finding of importance for any discussion of the neural control of the stretch reflex.

METHODS

Preparation. The experiments were performed on cats weighing 2–4 kg which were decerebrated under ether anaesthesia. The main results were obtained from seventeen preparations in which the stretch reflex was large and relatively constant over a long period. Eight of these were decerebrated by the anaemic method of tying the carotid and basilar arteries (Pollock & Davis, 1930). (Some of the results obtained on these particular preparations have already been described (Matthews, 1958).) Nine of the animals were decerebrated by transecting the mid-brain at or below inter-collicular level by suction, after tying the common carotid arteries. The level of section was deliberately made rather low in the hope of producing preparations with large tonic stretch reflexes, for when the tonic stretch reflex was small little active tension was developed on extending soleus slowly. In some preparations in which the stretch reflex was initially not large a further portion of mid-brain was removed by suction. In both kinds of preparations the forebrain was removed after decerebration, and the anaesthetic discontinued. The preparations usually breathed spontaneously throughout the experiment, but in a few cases they were ventilated artificially. Subsidiary results have been obtained from a number of other cats.

The right soleus muscle has been used throughout. It was separated from the distal part of gastrocnemius and the skin sewn over it again, leaving its tendon protruding. Its nerve, with that to the lateral head of gastrocnemius, was dissected free from the sciatic over several centimetres. The rest of the sciatic nerve, the hamstring nerve, the femoral nerve and the superficial branch of the obturator nerve were routinely divided (in the later experiments the deep branch of the obturator nerve and the lesser sciatic nerve were also cut). In about half the experiments the nerve to soleus was dissected out of the lateral head of gastrocnemius through which it runs, and all branches to gastrocnemius were cut. No systematic difference was then found, but this ensured that any electrical activity recorded arose from soleus and not from gastrocnemius. The foot was amputated as it was in the way of the myograph.

During myography the leg was held horizontal by steel pins at either end of the tibia, and the pelvis was steadied by pins in the iliac crests. The temperature of the soleus muscle was recorded with a thermocouple implanted nearby, and that of the rectum with a mercury thermometer. Both were maintained between 35 and 39° C (usually 36–38° C) by varying the amount of radiant heat falling on the preparation.

Myography and method of extending soleus. The tension in soleus was recorded by connecting its tendon by a hook (of steel except for a short plastic segment for insulation) to an isometric myograph. The myograph embodied an RCA 5734 mechano-electric transducer valve, the output of which was amplified and displayed on one beam of a double-beam cathode ray oscilloscope which could be photographed on slowly moving paper. The stretch reflex was elicited by moving the myograph while keeping the origin of soleus fixed. The myograph was mounted on a pivoted lever which could be moved at a slow constant rate by a screw thread driven by an electric motor. In the early experiments, which were on the cats decerebrated by anaemia, a series-wound motor was used. This tended to slow down slightly when it was opposed by a large stretch reflex, but the results do not appear to have been affected thereby, for the stretch reflexes studied were not sensitive to small variations in the rate of stretching. In the later experiments, on cats decerebrated by mid-brain section, a velodyne speed-controlled motor was used to ensure a constant speed in spite of a changing load on the motor (Williams & Uttley, 1946, Motor generator type 74). The velodyne was connected to the screw thread by reduction gearing and by an electromagnetic clutch. A large 'nut' was mounted on the screw thread and was constrained by rigid guides so that rotation of the screw thread was converted into a linear movement. This linear movement was transmitted to the lever on which the myograph was mounted by a piece of bicycle chain which was allowed to hang slack when the myograph and the muscle were in their initial 'resting' position. The motor and its clutch were mounted on a table separate from the rest of the apparatus in order to minimize the transmission of vibration to the preparation.

The rate of extension of the muscle was determined by recording the revolutions of the screw thread. During each revolution a cam on the same shaft as the screw thread momentarily closed a micro-switch, and allowed current to flow through a miniature neon light, giving a signal on the photographic paper immediately below the tension record. The beginning of the muscle stretch was signalled by a second neon light, directly above the first. This was automatically turned on when the electrical contact between the myograph and its stop (at its initial position) was broken as the myograph began to move after the slack in the chain had been taken up. It remained on throughout the time that soleus was being extended. In some experiments it was automatically turned off when the myograph made contact with the stop which limited the extension.

The apparatus allowed the soleus muscle to be extended at a constant and reproducible velocity while both its tension and the extension applied to it were continuously recorded. In most experiments soleus was stretched at a velocity of 1–2 mm/sec, but the rate could be varied from

0·5 to 6 mm/sec. Figure 1 shows a calibration of the apparatus obtained by using it to extend a coiled metal spring, with a compliance of the same order as that of soleus. The tension in the spring increased nearly linearly with the time while it was being extended, showing that it was being extended at a nearly constant velocity.

Electrical recording and stimulation. In some experiments the electrical activity of soleus was recorded between the insulated tendon hook and another electrode on the belly of the muscle. The second electrode was sometimes a needle in soleus, sometimes a silver plate lying on soleus. In each case a large amount of soleus lay between the recording electrodes. The electrodes were connected to a push-pull amplifier (frequency response limited from 50 c/s to 1·5 kc/s) and the output was displayed on the second beam of the cathode ray tube. In these experiments both heads of gastrocnemius were usually denervated.

Two stimulators were used to excite various nerves. One provided approximately exponential pulses of 0·5 msec time constant. The other gave square pulses of 0·1 msec duration. In each case the stimuli were delivered through a transformer connected to silver wire electrodes.

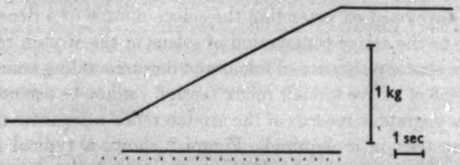

Fig. 1. Calibration of apparatus used for extending soleus. Above: myographic record of the increasing tension produced in a metal spring while extending it 15 mm at 2 mm/sec (initial tension 200 g, final tension 1400 g). Below: signal lights monitoring extension: upper light turned on at beginning of period of extension, and off when full extension applied; lower light flashed once for each 0·8 mm increase in the extension. (Signal light records retouched.)

Accuracy of measurements

Tension. Tension measurements were made directly from the photographic record on 70 mm recording paper. They were made to 0·2 mm, corresponding to a tension of 4–14 g depending upon the myographic sensitivity used. More accurate measurement did not appear necessary because the variability of the stretch reflex was often much greater. The zero of the myograph tended to drift, however, and the absolute value of the tension was not always determined as accurately as the changes in tension produced by extending soleus. When necessary small tensions found at the initial length of soleus could be recorded more accurately by adjusting the zero of the myograph and using a higher sensitivity than usual.

Extension. The initial length of soleus was set at the beginning of the experiment so that the tension developed in soleus at this length was under 50 g. The relation of this initial length of soleus to its normal range of extension will have been different in different preparations, for soleus will have been relatively shorter in experiments in which the rigidity was great (as in Granit, 1958). The final extension applied was usually 14 mm, but it was varied from 11 to 16 mm in different experiments. The passive tension developed in soleus at the final extension varied from 150 to 600 g. As the passive tension is approximately doubled for each 2–2·5 mm increase in the extension (see p. 524), it is probable that the final lengths of soleus in the different preparations agreed to within 5 mm. In many experiments soleus was probably extended slightly beyond its physiological range of movements, for it was found in separate experiments that extreme dorsiflexion of the ankle produced about 200 g of passive tension in soleus (range 100–400 g in four preparations).

During the stretching of soleus its extension was monitored by recording the revolutions of the screw thread. The tension in soleus was later measured from the record at each successive revolution of the screw thread, corresponding to each 0·8 mm increment of the extension (0·5 mm in earlier experiments). The zero of extension with respect to this scale was shown by the upper signal light (see Fig. 1). This measurement of apparent extension was accurate at least to 0·2 mm. No allowance, however, was made for the progressive yield of the myograph and its supports (under 0·1 mm/kg) or of the clamps holding the leg (under 0·05 mm/kg): but the greatest tensions recorded in the presence of the stretch reflex were under 2 kg, giving a maximum error of 0·3 mm at full extension. The compliance of the tendon was unknown, and may well have been larger than that of the myograph. Unfortunately when the tension in soleus was low (under 10–20 g) its extension could not be accurately determined. This arose because soleus and its tendon hook then lay slack, and might hang in a downward loop. Any extension applied to such a slack soleus would be partly expended in taking in the slack, rather than in actually extending soleus.

RESULTS

The passive tension developed on stretching soleus

The tension developed on extending the soleus muscle of a decerebrate cat is due not only to the active contraction of soleus in the stretch reflex, but also to the passive elastic resistance of soleus and its surrounding connective tissue. The component of active stretch reflex tension cannot be recorded alone and to interpret myographic records of the stretch reflex a separate determination of the passive tension is required. Figure 2 shows a typical record of the increase of passive tension produced by slowly extending a denervated soleus. During the stretching the tension developed in soleus increased progressively. When the stretching stopped and the extension was maintained the tension fell only slightly. In other experiments the rate of stretching was varied from 1 to 5 mm/sec and the tensions developed at a series of different extensions were compared for the different rates of extension. There was no systematic difference. It may be concluded that at the slow rates of stretching used the passive tension developed depended almost entirely upon the elastic properties of soleus, and very little upon its viscous properties.

The passive tension developed on stretching soleus increased exponentially with the extension when the passive tension was over 10–20 g. This is shown in Fig. 3, where the logarithm of the tension is plotted against the extension for the record of Fig. 2. A reasonably straight line is obtained with the tension doubling for each 1·9 mm increase in the extension. In seven other experiments the extension required to double the passive tension has varied from 1·9 to 2·8 mm (mean of 8 expts. 2·2 mm). This logarithmic relation has been previously obtained for the cat's soleus by using a series of extensions of different sizes (Banus & Zetlin, 1938; Eldred, Granit & Merton, 1953).

The active tension developed on stretching soleus

Figure 4 *a* is a typical record of the increase in tension produced by extending a soleus muscle with a large stretch reflex. Characteristically when the stretch reflex was present the tension did not increase quite smoothly as soleus was extended. The record itself provides a graph of tension against extension on a linear scale, for the extension increased linearly with time. At first the tension increased very little with extension and as the initial tension was under 10 g some 'slack' in the system may have been taken up initially. The line then curved upwards, until at larger extensions the relation between tension and extension became approximately linear. When the logarithm of the tension was plotted against extension, a straight line fitted the results obtained for extensions from 5 to 11 mm, but the whole curve was no longer fitted as was the case for the passive tension alone. The presence of the stretch reflex had transformed the extensibility of the muscle, so that tension was no longer exponentially dependent upon extension, and this was the usual finding when the stretch reflex was large.

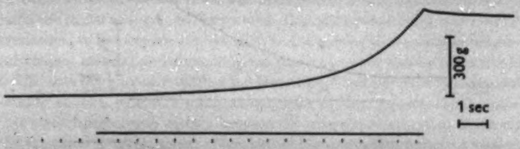

Fig. 2. The tension produced in a denervated soleus muscle by extending it 14 mm at 1·2 mm/sec. Above: myographic record of tension (initial tension under 10 g). Below: signal lights monitoring extension; upper light turned on at beginning of extension, and off when full extension applied; lower light flashed once for each 0·8 mm increase in the extension. (Signal light records retouched.)

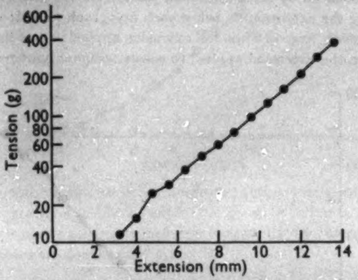

Fig. 3. The relation between the tension and extension for the denervated soleus of Fig. 2 plotted on a logarithmic scale for the tension and a linear scale for the extension.

The amount of active reflex tension developed by the stretch reflex for any particular extension was determined by subtracting the passive tension developed at the same extension. Figure 5 is a graph of active tension against extension during the extension of soleus shown in Fig. 4. As the passive tension formed only a small part of the tension developed when the stretch reflex was present, the form of the curve of Fig. 5 is rather similar to that of the record of Fig. 4*a*. Initially the tension increased approximately exponentially with extension, but for extensions over 8 mm the tension increased approximately linearly with extension, the slope being 150 g/mm. The initial curvature cannot be entirely attributed to the taking up of 'slack', for this should have been abolished by an extension of 2 mm when the tension was 20 g.

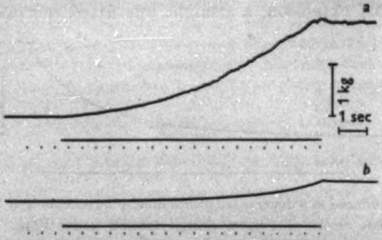

Fig. 4. Myographic records of the tension produced in the soleus muscle of a decerebrate cat by extending it 15 mm at 1·6 mm/sec. *a*, large stretch reflex present (initial tension under 10 g). *b*, stretch reflex abolished by being inhibited, passive tension alone remains. The signal light records monitoring the extension lie below each myograph record; upper light turned on at beginning of extension, and off when full extension applied; lower light flashed once for each 0·8 mm increase in the extension applied to soleus. (Signal light records retouched.)

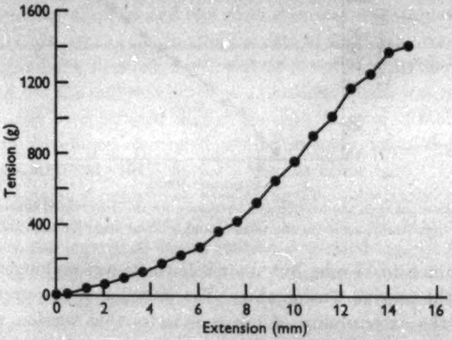

Fig. 5. The relation between the active tension in soleus and its extension obtained from the records illustrated in Fig. 4. Between 8 and 14 mm of extension the active tension increased by approximately 150 g for each mm increase in the extension.

The method of calculating the active tension assumes that the active and passive tensions sum arithmetically; but this is unlikely to be true even under rigidly isometric conditions. For it is probable that, when the muscle is under tension (active or passive), yield occurs in structures such as the tendon which lie in series with the contractile parts of the muscle. Thus the contractile parts of the muscle will be able to shorten slightly when they become active. Any passive elements in parallel with the contractile elements will thereby be shortened also, and will therefore contribute less to the myographically recorded tension than when the muscle is not contracting, but is held at the same over-all length. Subtraction of the passive tension, determined when the muscle is relaxed, from the total tension determined when the stretch reflex is present, will thus slightly underestimate the active tension developed by the stretch reflex. This error is unlikely to be appreciable when the passive tension is small in relation to the stretch reflex, but it may perhaps be significant when the stretch reflex is small. In addition, in all cases, yield of the tendon will make the extension applied to the muscle receptors slightly less than the extension applied to the whole muscle.

The passive tension has always been determined by denervation at the end of each experiment. In many experiments the passive tension was also determined, without sacrificing the preparation, by anaesthetizing the nerve to soleus with procaine. Complete nerve block was ensured by stimulating the nerve central to the anaesthetized region and observing that soleus no longer contracted. A still more convenient method of determining the passive tension was by reflex inhibition of the stretch reflex. This was done by stimulating the lateral popliteal nerve of the same side at 90/sec with shocks strong enough to evoke reflex movements of the opposite hind limb. Complete inhibition of the stretch reflex was certain because the irregular contraction of the stretch reflex was replaced by the smooth development of tension characteristic of the passive tension. When the electromyogram was recorded it provided another check on the completeness of the inhibition of the stretch reflex. These three methods of determining the passive tension (denervation, nerve block, reflex inhibition) agreed well.

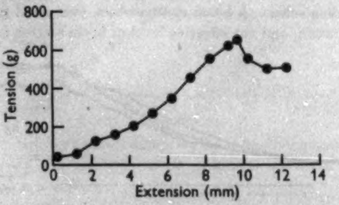

Fig. 6. A graph of active tension against extension from another preparation. The active tension failed to increase progressively with the extension, possibly owing to autogenetic inhibition of the soleus motoneurones occurring at the larger extensions. (Soleus extended at 1·6 mm/sec for 12 mm; final passive tension was 340 g.)

Inhibition. In most experiments the active tension increased continuously during the progressive extension of soleus. In a few experiments, however, the active tension did not increase at the end of the period of extension when the passive tension was increasing rapidly. An example is shown in Fig. 6, where, after a progressive increase in the active tension during the first 10 mm of extension, the active tension fell by 150 g while the extension was increased from 10 to 12 mm. The total myographically recorded tension was approximately constant, though rather irregular, from 10 to 12 mm extension, so that the fall in the active tension corresponded approximately to the increase in the passive tension. In some other experiments the active tension did not fall but became constant in spite of continued extension. In these experiments the myographically recorded tension continued to increase with the extension, for the passive tension continued to increase. The occasional failure of the active tension to increase throughout the extension has, in most cases, been tentatively ascribed to inhibition of the soleus motoneurones by impulses from the proprioceptors of soleus (autogenetic inhibition).

An alternative explanation for the failure of the active tension to increase with extension is that some part of the reflex arc had become 'saturated'. In the experiment of Fig. 6 this is unlikely, as the effect was variable and later in the experiment larger active tensions were developed at the same extensions. Also in this case the active tension was limited to only 700 g, while the maximal tension developed by tetanic stimulation of the nerve to soleus at 90/sec was 2·1 kg. In a few experiments, however, saturation of some part of the reflex mechanism has been suspected. In these cases the active tension increased to 70–80% of the tension developed by maximal stimulation of the nerve to soleus at 90/sec at the extended length. This level of active tension was a relatively constant ceiling even when excitatory influences in addition to the stretch reflex were brought to bear upon the soleus motoneurones. Stimulation at 90/sec will probably over-estimate the contractile power of soleus available physiologically, as the soleus α motoneurones normally discharge at much lower frequencies. Both saturation and inhibition might have been seen more frequently if soleus had been extended further, but extensions producing over 500 g of passive tension were avoided for fear of producing irreversible changes.

Complete disappearance of the active tension with increase in the extension of soleus (clasp-knife phenomenon) has been seen only once in the present series of experiments. In this preparation flexor rigidity was as marked as extensor rigidity, and little more than 100 g of active tension was developed on extending soleus. A blood clot had been evacuated from low in the mid-brain some time after decerebration, and the effective level of brain section is unknown.

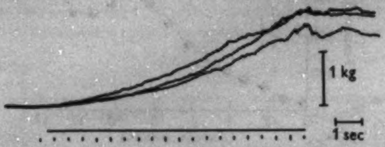

Fig. 7. Superimposed tracings of three myographic records from the same experiment which illustrate the variability of the stretch reflex. Soleus extended 15 mm at 1·6 mm/sec. The middle record is that of Fig. 4*a*. The upper and lower records were selected from the 10 records taken in the 20 min afterwards to show the maximum spontaneous variation of the stretch reflex found in that time. Below: signal lights monitoring extension: upper light turned on at beginning of extension, and off when full extension applied; lower light flashed once for each 0·8 mm increase in the extension applied to soleus. (Maximum error in superposition of signal lights less than 0·2 mm of extension.)

Variability. Attempts to describe the relation between tension and extension mathematically have been obstructed by the variability of the stretch reflex. Figure 7 illustrates the degree of spontaneous variability found in a single preparation. The three records differ in the rate at which the stretch reflex increases with tension, and in the smoothness with which it increases. In some experiments the correspondence between successive records was better, in some worse. The variability was certainly caused by changes in the stretch reflex and not by changes in the passive properties of the muscle, for repeated determinations of the passive tension alone differed very little (within the error of measurement). The causes of the variation in the stretch reflex have not. however, been clearly defined.

Linearity of the relation between active tension and extension

A linear relation between active tension and extension usually fitted the experimental results once the active tension had increased above 200–400 g. At lower tensions a curve whose slope increased with the extension, or another straight line of less slope than that found at higher tensions, was usually required (cf. Granit, 1958). On occasion, however, a straight line fitted the experimental points over a very wide range. Such a result is shown in Fig. 8, where the active tension increased by 75 g for each millimetre of extension throughout almost the whole range of the 15 mm extension studied. Other results obtained in the same experiment were generally similar for extensions up to 12 mm, but showed a slight deviation from the linear relation for the last 1–3 mm of extension, where the slope of the line joining the points fell. In general, in view of the variability of the stretch reflex, a straight-line relation between active tension and extension offers a reasonable approximation and permits comparison of the stretch reflexes obtained in different preparations.

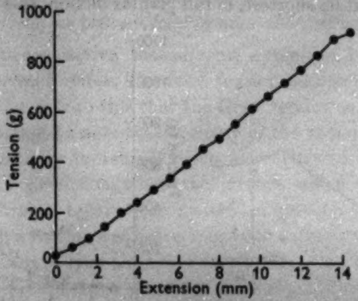

Fig. 8. A graph of active tension against extension from another preparation in which a linear relation was found over a wide range of extensions. (Soleus was extended 14 mm at 1·6 mm/sec, the final passive tension was 370 g.)

Value of slope. The slopes of the approximately linear portions of the curves of active tension against extension have been compared for the seventeen preparations which were studied in detail. The maximum value of the slope found in any one experiment was used, provided that it applied over at least 4 mm of extension, and provided that it was found repeatedly. In these preparations the mean value of the maximum slope was 90 g/mm, with a range of from 40 to 200 g/mm in different preparations. These values are similar to those of Granit (1958).

A more physiological measure of the tension is to express it in terms of the active tension developed by a maximal contraction of the muscle. This was determined by stimulating its nerve at 90/sec with shocks supra-maximal for the large motor fibres. The determination was made with soleus extended because the maximal slope for the increase of tension in the stretch reflex was usually found as the extension of soleus was nearing completion, and because the tetanic tension may increase as soleus is extended. The maximal tension ranged from 1·4 to 3·0 kg in different preparations. The slope for each experiment was then expressed as a percentage of the tetanic tension for that experiment. The mean value of the slope was then found to be 5·1 % of the tetanic tension per millimetre increase in the extension. As the belly of soleus is about 5 cm long at its shortest, in full plantar flexion, this corresponds to the production of about 2·5 % of the tetanic tension for a 1 % increase in the length of soleus. (Expressing the tension in terms of the tetanic tension did not decrease the scatter found between different preparations, which ranged from 2·3 to 11·5 % of the tetanic tension per millimetre increase in the extension.)

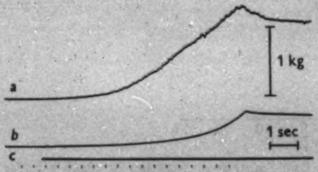

Fig. 9

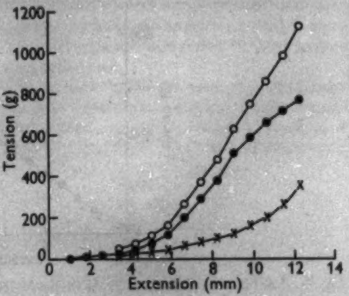

Fig. 10

Fig. 9. Myographic record showing a linear relation between total tension and extension when the stretch reflex was present. *a*, tension recorded when stretch reflex was present. *b*, passive tension recorded when nerve was completely anaesthetized with procaine. *c*, signal light records applicable to both *a* and *b*; upper light turned on at beginning of extension (end of period of stretching not signalled, as previously); lower light flashed once for each 0·8 mm increase in the extension. (Signal light records retouched. Soleus was extended 13 mm at 1·9 mm/sec.)

Fig. 10. Graphs of tension against extension derived from the records illustrated in Fig. 9. Plotted from above downwards are the total (○), the active (●) and the passive (×) tensions developed on extending soleus. In this case a straight line fits the points for total tension better than those for the active tension.

Of the seventeen preparations compared eight were decerebrated by anaemia and nine by mid-brain section. The mean value of the slope was similar in the two cases, being 88 g/mm for the anaemic decerebrates and 93 g/mm for the ordinary decerebrates. (The range in each case was also similar, being 45–200 g/mm for anaemic decerebrates, and 40–175 g/mm for the cats decerebrated by mid-brain section.) When the slope was expressed as a percentage of the tetanic tension there was again a slight, but insignificant, difference between the two types of preparation. This comparison, therefore, suggests that the factors determining the maximum slope are similar in the two preparations, though they differ in certain other respects (Granit, Holmgren & Merton, 1955). It should be noted, however, that the cats decerebrated by mid-brain section may not have been typical of all preparations so decerebrated, for the level of section was usually low, and they mostly had large, well-maintained stretch reflexes.

The relation between total tension and extension

The relation between active tension and extension is not a complete description of the stretch reflex. The total tension (active plus passive) must also be considered, for it is to this that the Golgi tendon organs are sensitive. It is therefore important to note that in many of the present experiments the total tension (as actually measured) often also followed an approximately linear relation with extension when the stretch reflex was present. The variability of the stretch reflex often made it impossible to decide whether total tension or active tension was better related to extension by a straight line. In both cases the rule usually applied over only a part of the range of extensions studied. When the stretch reflex was large in relation to the passive tension, the active tension and the total tension were approximately the same, and it was under these conditions that a linear dependence upon extension was found best for either. When the stretch reflex was smaller the graph of total tension against extension usually curved upwards, corresponding to the upward curvature of the passive tension. The graph of active tension against extension was then better fitted by a straight line than that of the total tension. In a few cases, however, the graph for total tension was more nearly linear than that for active tension. An example of this is shown in the myographic records of Fig. 9. There the total tension increased approximately linearly with extension in the later stages of extension, even though an increasing proportion of it was contributed by the passive tension; this was common in this preparation. Figure 10 shows the same results plotted, and provides a more direct comparison of the dependence of the active and the total tension upon the extension. The slope of the line joining the points for active tension decreases at larger extensions, while that for the total tension persists unchanged. This flattening of the active tension curve may perhaps, as already discussed, be attributable to autogenetic inhibition. If so, in the present case, the inhibition has produced a tension deficit in the active tension corresponding to the increase in the passive tension. Whenever 'autogenetic inhibition' appeared to be present the flattening of the active tension curve tended to improve the linearity of the total tension curve, almost as if this were the 'purpose' of the inhibition. Sometimes, however, the inhibition caused the slope of the curve of total tension against extension to fall off at large extensions.

The response to sudden stretch

The response of soleus to a relatively sudden stretch has been observed as well as its response to a slow stretch. The sudden stretch was applied by moving the myograph by hand instead of by the electric motor. By this means an extension of 10–15 mm was applied in about 0·5 sec instead of in the 2–3 sec minimum for the slow stretch, but the sudden extension was no longer applied smoothly at a uniform velocity. The response of a denervated soleus to an extension of 13 mm suddenly applied and then maintained for 3 sec is illustrated in Fig. 11 *a*. This shows that the passive tension produced by a sudden extension fell very little with time, and this was the invariable result.

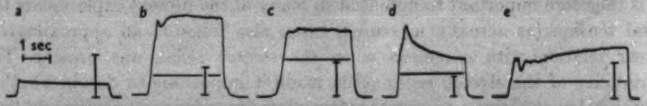

Fig. 11. Myographic records of the tension produced in soleus by a stretch applied suddenly (in about 0·5 sec) and then maintained for 3–4 sec. Records obtained from different preparations to illustrate the variation in the degree of maintenance of the stretch reflex with time. All records on the same time scale, but on different tension scales (500 g calibration bar for each record). *a*, response of a denervated soleus; *b*, *c*, *d* and *e* stretch reflex present. The passive tension developed by each extension is shown by a horizontal line. In all cases the initial tension was under 50 g; the extensions applied range from 13–15 mm.

In the innervated soleus the tension usually fell further after the completion of stretching than did the passive tension alone. Frequently, however, the stretch reflex decayed very little while the extension was maintained (tonic stretch reflex). An example is shown in Fig. 11 *b*, obtained from a cat decerebrated by anaemia. This method of decerebration usually produced preparations with well maintained stretch reflexes. Cats decerebrated by mid-brain section also frequently had well maintained stretch reflexes (Fig. 11 *c*). Sometimes, however, in this type of preparation the stretch reflex decayed fairly rapidly to a more or less steady plateau (Fig. 11 *d*). When there was much decay in the tension produced in response to a sudden stretch, little active tension was developed on slow extension of soleus. In this case, the adjustment of the stretch reflex appeared to be occurring during the slow stretching, and the stretch reflex could not then be studied by this method. In general when the stretch reflex was large it was also well maintained, and most of the present investigations have been upon preparations with such reflexes. The various results obtained, therefore, apply to the 'tonic' rather than to the 'phasic' component of the stretch reflex.

An unusual response to a sudden stretch is illustrated in Fig. 11 *e*, where the tension increased slowly throughout the 4 sec for which the extension was maintained, although the actual stretching was complete in under 1 sec. This record was obtained from a cat decerebrated by anaemia, but similar records have occasionally been obtained from cats decerebrated by brain section. This slowly augmenting response occurred usually after the preparation had been left undisturbed for a period (no stretching or nerve stimulation). Also a further stretch applied immediately after the muscle had been allowed to relax again usually elicited a larger response, but this no longer augmented as before. Further stretches then produced approximately constant responses. This potentiating effect of successive stretches has been fairly common in the present series of experiments and frequently occurred without the augmenting response.

The small fall in the tension seen at the beginning of the stretch reflexes of Fig. 11 *b* and *e* was common. In some experiments the end of the extension was signalled by turning off a signal light when the myograph reached its stop. It could then be seen that the dip occurred after completion of the extension and was not due to an irregular pull. When the electromyogram was recorded the dip was found to be accompanied by a brief silent period. Its time course was not studied, however, as the method of stretching soleus rapidly by hand did not appear sufficiently controlled or repeatable.

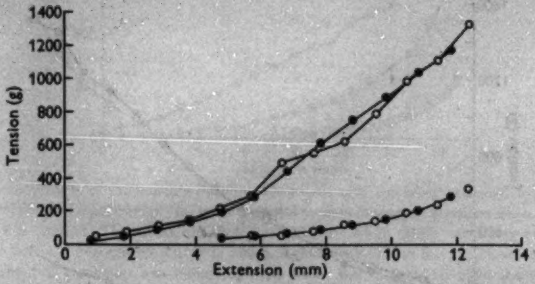

Fig. 12. A comparison of the tension–extension curves obtained by stretching soleus slowly (●, soleus extended at 1·3 mm/sec), and by applying the extension suddenly as in Fig. 11 (○). Upper points obtained when the stretch reflex was present (total tension). Lower points obtained when the stretch reflex was abolished (passive tension). The stretch reflex elicited by a sudden extension was well maintained with time, its height was measured 1·5 sec from the beginning of the extension. (Same experiment as Fig. 11 *b*.)

In some experiments sudden stretches have been used to determine the tension–extension curve of the stretch reflex. Extensions of different lengths were applied to soleus, and the tension produced by each was measured after the reflex had reached a fairly steady level. This method has been used by Granit (1958). Figure 12 shows that in a preparation with a well maintained stretch reflex it gives the same result as the method of slow stretching. This has also been found in another nine experiments, in which the stretch reflex was also well maintained with time (preparations with poorly maintained stretch reflexes have not been studied in this respect).

The effect of varying the rate of stretch

In preparations with well maintained stretch reflexes, varying the rate of extension had little or no effect on the tension developed at any particular extension. An example of varying the rate of extension fivefold is shown in Fig. 13. The upper points show the total tension developed on extending soleus at 0·5 mm/sec (●), and at 2·7 mm/sec (○) when a large stretch reflex was present. In other experiments it had been confirmed that varying the rate of stretch had no effect on the passive tension developed. It may be concluded therefore that in this experiment it had no effect on the active tension either. In three other preparations no difference has been produced by varying the rate of extension from 1 to 6 mm/sec. In several others a smaller range of velocities has been tested without effect.

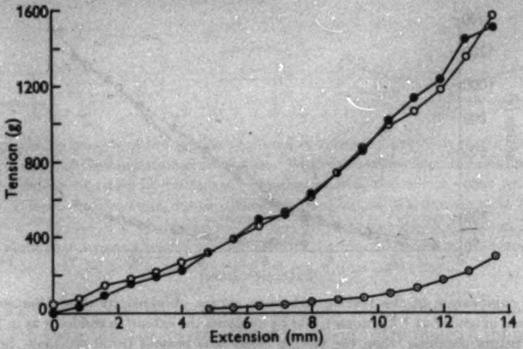

Fig. 13. A comparison of the tension–extension curves obtained by extending soleus at two different rates. Upper points show the total tension recorded when the stretch reflex was present, and soleus was extended at 2·7 mm/sec (○), and at 0·5 mm/sec (●). Lower curve (⊙) shows the passive tension produced when soleus was extended at 1·2 mm/sec while the stretch reflex was inhibited. The preparation, which had been decerebrated by mid-brain section, had a well maintained stretch reflex in response to an extension applied suddenly.

When the stretch reflex in response to a sudden stretch was not well maintained, then after the completion of slow stretching there was usually also a slight decline in the tension, to a lower plateau. In such cases faster stretches might be expected to produce a greater tension at any particular extension than slower stretches. The results of two experiments were in accordance with this, but the difference found was too small to show whether the change in velocity had altered the form of the tension–extension relation in any simple manner (e.g. by altering primarily the slope or the intercept of the linear portion of the curve).

The effect of releasing soleus

The tension developed by the stretch reflex while a muscle is being released is of as much physiological interest as that developed while a muscle is being stretched. With the present apparatus soleus could be released at a constant velocity by reversing the electric motor used to extend it. Records taken on stretching and releasing a spring showed that the system of monitoring lights gave an accurate measure of the extension while it was being released, and errors in measurement of length between stretch and release were under 0·2 mm. A typical result of an experiment on soleus is shown in Fig. 14. The lower curves (○) show the behaviour of the passive tension, the upper points being obtained on stretching soleus and the lower points on releasing it. The passive tension on release was about 40 g below that found at the same extension when soleus was being stretched. The upper curves of Fig. 14 (●) show the total tension developed in soleus when the stretch reflex was present. The points obtained by stretching soleus lie above those obtained while releasing it. The difference between these stretch and release curves is much greater than that between the similar curves for the passive tension, and is thus mainly due to the difference between the active tensions during stretch and release. Part of the difference is due to the slight fall of tension which occurred in the 2 sec after the completion of stretching while the final extension was maintained and before the release started. This is shown by the two points at the right-hand end of the curves. In addition the tension fell more rapidly for the first few millimetres of release than it had risen for the last few

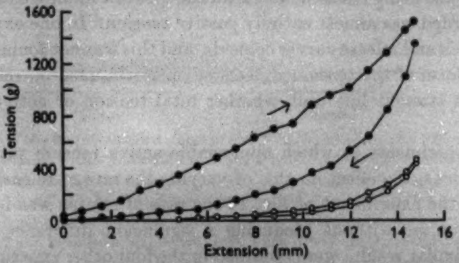

Fig. 14. A comparison of the tensions developed in soleus as it was being extended, and as it was being released. Upper curves (●) show the total tension developed in soleus when the stretch reflex was present. The ascending limb was obtained while soleus was being extended at 1·7 mm/sec; the descending limb was obtained when soleus was being released at the same rate. The full extension was maintained for 2 sec, and during this time the tension fell slightly. The lower curves (○) show the passive tension alone, obtained similarly when the stretch reflex was inhibited. The active tension developed at any extension of soleus was greater when soleus was being stretched than when it was being released (hysteresis).

millimetres of extension. For extensions of soleus between 7 and 12 mm the stretch and release curves lay approximately parallel.

Some difference between the stretch and release curves has been found in seven out of eight experiments in which a graphical comparison has been made. It occurred even when no fall of tension developed while the final extension was maintained. It has also been seen by direct inspection of the experimental records in many other experiments, and occurs in cats decerebrated either by mid-brain section or by anaemia. The absolute difference between the two curves varied greatly from experiment to experiment. When the stretch reflex was small or when it was poorly maintained, very little active tension persisted while soleus was being released, and after the first few millimetres of release the tension recorded was almost entirely passive tension. In one experiment only did the stretch and release curves coincide, and this was not found in all records taken. The form of the tension–extension curve obtained on release of soleus did not fit a straight line well, whether total tension or active tension was considered.

In five experiments in which appreciable active tension persisted during release of soleus, the effect on this of varying the rate of releasing soleus was studied. In the experiment of Fig. 14 the rate of release was increased from 1·7 to 5·7 mm/sec without producing a significant difference in the curve obtained. Similar results were obtained in the four other experiments. Nor in these experiments did the rate of stretch affect the tension developed on extending soleus. Thus, in the range of velocities studied, the difference between the tension–extension curves obtained on stretch and release appears to be dependent upon the direction of movement, and not upon the absolute value of the velocity. The difference between stretch and release is a hysteresis effect, for at any particular tension the length of soleus was greater when it was being released than when it was being extended. Similar hysteresis effects have been found on stretching and releasing the forelimb muscles of cats decerebrated by anaemia (Pollock & Davis, 1929).

Electromyography during release. Several factors probably contribute to the difference between the curves found on stretching and releasing soleus. One likely cause is that muscle spindle afferents might be expected to discharge at a lower frequency for a given extension when soleus is being released than when it is being extended (cf. B.H.C. Matthews, 1933). In addition there is the fact that the tension developed by a muscle with a constant degree of activation depends upon how fast the muscle is changing length, and whether it is shortening or being extended (force–velocity relation of muscle, recently reviewed by Wilkie, 1954). That the force–velocity property of muscle is not the only factor concerned has been shown by recording the electrical activity of soleus at the same time as its tension. It was then found that, like the tension, the amount of electrical activity recorded on stretch and release of soleus was also asymmetrical. An example of this is shown in Fig. 15. The electromyogram was obtained by placing one recording lead on the belly of soleus and the other upon its tendon, thereby probably picking up the electrical activity of most of the motor units which were active at any one time. (It was certain that the electrical activity was recorded from soleus, for no other muscle in the lower part of the leg remained innervated. When the nerve to soleus was cut the electrical activity produced on stretching soleus was abolished.) In Fig. 15 there was no electrical activity recorded and no active tension developed at the initial length of soleus. On extending soleus, electrical activity was first recorded when soleus had been extended by 3 mm. It then increased progressively to the end of the period of extension, and remained approximately constant while the extension was maintained. On releasing soleus the electrical activity decreased rapidly and at any particular length of soleus it was less than it had been while soleus was being extended. This is most readily seen by comparing the ascending and the descending records at the two points marked by arrows, which correspond to the same extension of soleus from its initial length. At this extension, when soleus was being extended there was marked electrical activity, while when soleus was being released there was none. These findings have been frequent, and show that the hysteresis effect is not due solely to the contractile properties of the extra-fusal muscle fibres of soleus.

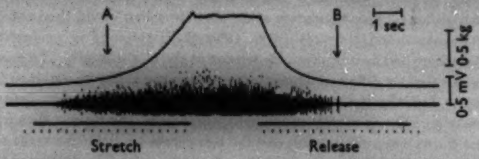

Fig. 15. Records of the tension in soleus (above) and of its electrical activity (below, recorded with belly-tendon leads) to show the difference found on stretching and releasing soleus. Signal lights at bottom monitor the extension; upper light turned on at the beginning of movement, and off at the end of movement; lower light flashed once for each 0·8 mm change in the extension (signal light records retouched). The arrows are drawn to indicate an extension of soleus of 7 mm from its initial length, both when it was being stretched and when it was being released. (Total extension, 14 mm; rate of extension, 2·8 mm/sec; final passive tension, 550 g, which formed about half the total tension in this case. The electrical record was cut off abruptly at the bottom of the record because it then fell out of the field of view of the camera.)

Further observations on electromyography. The electrical record obtained with large electrodes is also of interest because it provides a measure of the total amount of motor discharge to soleus, which is independent of the variation of the contractile strength of soleus with variations in its length. In the present experiments there has been no attempt to make the recording quantitative, as might be done by integrating the electrical record (cf. Bigland & Lippold, 1954). Three observations are, however, of interest. First, the electrical discharge of soleus was most usually found to increase progressively as soleus was extended, and often the recruitment of new motor units could be seen. This is in confirmation of Denny-Brown (1929*a*) and of Granit (1958). Secondly, when only slight active tension (10–40 g) was developed at the initial length, the electrical activity nevertheless sometimes involved too many motor units for their individual discharges to be distinguished with a recording speed of 1 cm/sec. This suggests that the muscle fibres were contracting in unfused tetani, as the fused tetanic tension of a single motor unit is probably about 12 g (Granit, 1955). This is in agreement with observations on the slow rate of firing of motor units of soleus in the stretch reflex (Denny-Brown, 1929*a*). Thirdly, when the electrical discharge at the initial length was marked it did not always increase very much as soleus was extended, though the muscle then developed an increasing active tension (cf. Granit, 1958).

After-effect of stretching. The state of soleus and its reflex centres was sometimes altered for a time after a prolonged stretch had been released. In some cases soleus hung slack at the initial length after being released, whereas it had been developing slight active tension before being extended. A few tendon taps or stimulation of the nerve to soleus would then usually 'tighten up' soleus again, as was also found by Granit (1958). Often the 'tightening up' occurred spontaneously, particularly when there had been appreciable tension at the initial length. If soleus were extended when it was slack a slightly different tension–extension curve might be obtained from that found when it was taut. Initially the tension increased hardly at all, probably because the applied extension was taking up the slack rather than extending soleus. The tension then rose more rapidly than it did when soleus was initially taut, and the two curves tended to converge so that there was little difference in the stretch reflex developed at large extensions. The difference between the two conditions has only rarely been greater than the spontaneous variations due to unidentified causes. In studying the effect of various other influences on the stretch reflex the tension–extension curves which were compared were obtained under similar conditions with regard to previous stretch (e.g. after a prolonged stretch, or after tendon taps).

The effect of extension of soleus on the tension developed on stimulation of its motor nerve

Extension of a muscle within the physiological range normally increases its strength of contraction, and this might be expected to play a part in the stretch reflex (e.g. Granit, 1958). The effect of extending soleus on the tension developed in a maximal fused motor tetanus has, therefore, been recorded throughout the present series of experiments. After cutting the nerve to soleus at the end of the experiment (to determine the passive tension) its peripheral end was placed on stimulating electrodes. It was then stimulated at 90/sec with shocks supramaximal for the motor fibres, first with soleus at the initial length used in the experiment and then with soleus extended by the amount used to elicit the stretch reflex. The tension developed at the initial length varied from 1 to 2 kg in all but one of the different preparations. When soleus was extended the tension was increased by 5–55 %. If, however, soleus was allowed to shorten by 1–2 cm below its initial length, the tension developed decreased very greatly, to a small percentage of its original value. (In one experiment the tetanic tension developed at the initial length was only 200 g, and increased to 1600 g when soleus was extended by 13 mm; and it is probable that in this case the initial length was set much lower than usual.)

Unfused tetanic contractions. In contrast, the effect of extending soleus on the tension produced by stimulating its nerve was found to be large for unfused tetanic contractions. Figure 16 *a* shows the tension produced in soleus on stimulating its nerve at 8/sec, first with soleus at the initial length, and then with soleus extended. When soleus was at the initial length the tension produced was barely greater than that produced in a twitch (the first response is small because the muscle was lying slack). When soleus was extended, stimulation at 8/sec produced an unfused tetanus which developed about twice the twitch tension. Figure 16 *b* shows that when the contraction was a fused tetanus the active tension increased by only 5 % when soleus was extended.

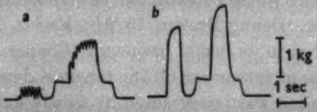

Fig. 16. The effect of extension of soleus on the tension developed in it on stimulation of its motor nerve at 8/sec (*a*) and at 60/sec (*b*). In each pair of records the nerve to soleus was stimulated with maximal shocks, first with soleus at the initial length used earlier in the experiment for studying the stretch reflex, and then after soleus had been extended by the amount used to elicit the stretch reflex (14 mm extension, note increase in passive tension, nerve cut centrally). Extending soleus had little effect on the active tension developed in the fused tetanic contraction, but greatly increased the tension developed in the unfused contraction.

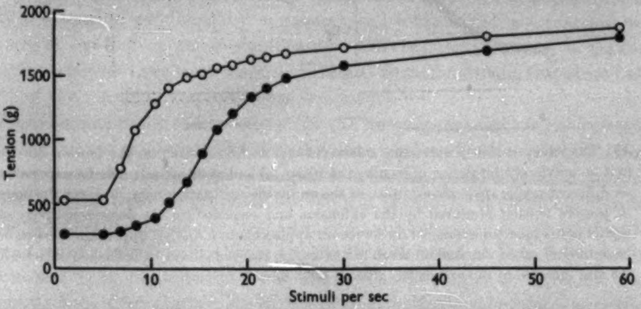

Fig. 17. The effect of the frequency of stimulation of its motor nerve on the tension developed in soleus. The lower curve (●) was obtained with soleus at the initial length used for studying the stretch reflex. The upper curve (○) was obtained with soleus extended by 14 mm (passive tension then 400 g). When soleus was extended a tetanic contraction was produced by a lower frequency of stimulation than when soleus was at the initial length. Same experiment as Fig. 16, which shows two pairs of the results used to determine the full curve. The increase in tension (active tension) produced by stimulation was measured at the peaks of the individual waves at 0·5 sec from the beginning of stimulation. (Muscle temperature 37 ± 1° C.)

Figure 17 shows the effect of extension on the tension developed at the two lengths of soleus for a number of different frequencies of stimulation. At low frequencies of stimulation (7–20/sec) the tension developed increased greatly when soleus was extended, but at higher frequencies of stimulation extension of soleus had little effect on the tension developed. When soleus was extended a much lower frequency of stimulation was required to produce a given fraction of the maximal fused tetanic tension than when soleus was at its initial length. Similar results were found in other preparations. These effects may be interpreted as due to a decrease in the fusion frequency of soleus when it is extended. This would be expected, as stretching soleus increases the duration of its twitch (Denny-Brown, 1929*b*; Eccles, Eccles & Lundberg, 1958) and this is related to its fusion frequency (Cooper & Eccles, 1930).

The effect of a range of extensions on the tensions developed with different frequencies of stimulation was investigated by slowly extending soleus at the same time as its nerve was being stimulated. An example is illustrated in Fig. 18. With a low frequency stimulation (*a*) little tension was developed at the initial length, but the tension increased rapidly as soleus was extended.

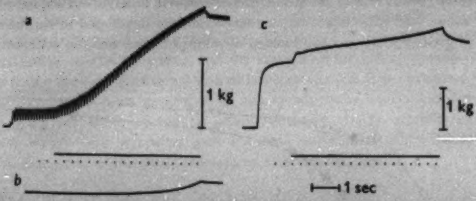

Fig. 18. The effect of slowly extending soleus (14 mm at 2·8 mm/sec) on the tension developed in it on maximal stimulation of its nerve at 10/sec (*a*) and at 30/sec (*c*). *a* and *c* were recorded on different myographic sensitivities, as shown by the calibration bars. *b* shows the increase in passive tension produced by the extension and recorded on the same sensitivity as *a*. Signal lights monitor extension (same record applicable to *a* and *b*); upper light turned on at beginning of extension, and off when full extension reached; lower light flashed once for each 0·8 mm increase in the extension. (Signal light records retouched.)

On stimulating at 30/sec (*c*) a large tension was produced at the initial length, and it increased less, both relatively and absolutely, with extension. In addition, in Fig. 18*a* the amplitude of the tension excursion following each stimulus progressively decreased as soleus was extended, showing that the contraction was becoming more of a fused tetanus as well as developing a greater tension. (Figure 18 also shows that throughout the period of stretching a larger tension was developed at each extension than when soleus was maintained at a constant length, as has been shown for other muscles (e.g. Abbot & Aubert, 1952).) These effects of progressive increase in extension have been confirmed by applying short bursts of stimuli (about 1 sec duration) while soleus was being extended at about 0·5 mm/sec. By both methods it was found that variation of the rate of stimulation might alter the form of the curve of tension against extension. With higher frequencies (nearly fused tetani) an approximate plateau of tension was frequently found for the larger extensions. With lower frequencies the tension might increase throughout the range studied.

Submaximal contractions of soleus. The results so far described were obtained using maximal stimulation of the nerve to soleus. Similar results have also been obtained when only a part of soleus was contracting. Two different ways have been used to excite soleus submaximally. In some experiments the strength of the stimulus to the nerve was adjusted so as to excite only a proportion of the motor fibres supplying soleus. In this case it proved difficult to ensure that a constant number of motor fibres were excited as the muscle was extended. Additional experiments were therefore performed in which the peripheral end of a portion of an appropriate cut ventral root was stimulated, instead of the nerve to soleus. (These five cats were anaesthetized with sodium pentobarbitone, instead of being decerebrated. In three of them soleus was completely isolated from gastrocnemius. Only the last 15 mm of the physiological range of extension was investigated, for as is discussed below this appeared to correspond to the range of extensions over which the stretch reflex was present in the decerebrate cats.) Both kinds of experiment showed that the tension produced by a submaximal tetanus did not increase with extension by a proportion significantly different from that of a maximal tetanus, whether they were fused or unfused. Stimuli eliciting responses below 10% of the maximal response were not used.

Physiological range of extensions. In the five experiments on anaesthetized cats the maximal motor response of soleus was studied in an approximate manner throughout the range of lengths which soleus could take up in the body. These lengths were determined by tying a thread to the tendon of soleus while it was still attached to the calcaneum, and recording the position of this thread, with respect to the tip of the fibula, with the foot both fully plantarflexed and fully dorsiflexed at the ankle. The full range of movement was about 23 mm. Over the last 10–15 mm of this physiological range of extensions the tension developed in a maximal fused tetanic contraction of soleus altered relatively little ($<30\%$), though the fusion frequency of soleus decreased as the extension was increased. (This limited range of extensions therefore appeared to correspond roughly with that over which the stretch reflex has been studied in the decerebrate cats, and this was confirmed by the similarity of the passive tensions in the two cases.) In contrast, over the first 8–13 mm of the physiological range of extension the fused tetanic tension increased greatly with extension. Soleus at its shortest physiological length developed only 20–30% of the tension it developed at its longest, even when the frequency of stimulation was high (60/sec or over) and the contraction was a smooth tetanus in both cases. When the frequency of stimulation was low ($<$ 20/sec) the contraction of soleus was sometimes barely detectable when soleus was at its shortest physiological length. Thus with the foot in full plantar flexion soleus was surprisingly ineffective at developing tension, and it may be questioned whether soleus ever functions usefully at this length in the intact animal.

DISCUSSION

The present experiments and those of Granit (1958) show that the active tension developed in the stretch reflex of soleus is often approximately linearly related to the extension. The records of Liddell & Sherrington (1924, 1925) on the quadriceps, and of Denny-Brown (1929*a*) on soleus, may also in some cases be similarly interpreted, as has already been noted by Mountcastle (1956). In the present experiments an approximately linear relation also held between the 'total' tension and the extension, and it has not been possible to decide whether one or other of these two relations provides a better description of the stretch reflex. At any rate, whatever the 'true' relation determining the properties of the stretch reflex, it is likely to be governed by the behaviour of the primary endings of the muscle spindles, for it is probably their excitation by extension of the muscle which initiates the stretch reflex. It is therefore of interest that in the decerebrate cat, with virtually intact motor and sensory supply to the muscle studied, the frequency of discharge of these endings is linearly related to the extension when a study level of discharge has been reached (Eldred *et al.* 1953; Granit, 1958). A similar relation may be assumed to have existed during the course of the slow progressive extensions applied in the present experiments, for the results obtained depended upon the applied extension rather than upon the rate of extension.

The results obtained when using soleus as a nerve-muscle preparation unfortunately suggest that it is impossible to deduce the amount of efferent discharge elicited in the stretch reflex from a knowledge of the active tension in soleus. For the tension produced in soleus by stimulating its nerve at low frequencies (5–20/sec) increased markedly as soleus was extended, and the frequency of discharge of soleus motoneurones is similarly low when they are excited in the stretch reflex (Denny-Brown, 1929*a*; Granit, 1958). The contribution of this peripheral effect to the production of tension in the stretch reflex is difficult to assess; for extension of soleus only slightly increased the tension developed in a fused tetanic contraction when this was studied in the range of extensions in which the stretch reflex was found earlier in the same experiment. The relative proportions of muscle fibres contracting in fused and in unfused tetanic contractions is unknown. It may be concluded, in agreement with Granit (1958), that the peripheral properties of muscle might be of great importance in the development of tension in the stretch reflex. Granit, however, did not distinguish between fused and unfused tetanic contractions, and in contrast to the present results it appears that he usually found large changes in the fused tetanic tension of soleus as it was extended. It appears probable that Granit studied soleus at lengths shorter than those at which the stretch reflex was found in the present experiments, for he recorded a 'nearly maximum' tetanic contraction which developed only 150 g tension at the initial length of soleus (his Fig. 6, stimulation at 48/sec), whereas tensions of 1–2 kg have been usual in the present experiments.

Because of the complication introduced by this variation of the fusion frequency of soleus, the apparently simple relation between the tension in soleus and its extension cannot readily be explained. Indeed, either of two rather different kinds of central reflex mechanisms might explain the present results. First, it is possible that the reflex centres transform excitatory afferent impulses into efferent impulses by some simple rule. Secondly, it appears equally possible that the stretch reflex results from a 'balance' between the inhibitory impulses and the excitatory impulses converging on to the 'reflex centres' of soleus as a result of its extension (cf. Denny-Brown, 1929 *a*). The view that excitatory impulses alone are of importance is favoured by work showing that inhibitory reflexes acting on extensor muscles are suppressed in decerebrate cats (Job, 1953; Eccles & Lundberg, 1958). It is therefore possible that autogenetic inhibition is unimportant in decerebrate cats, and that the approximately linear relation between active tension and extension must be explained in terms of the input-output relations of the spinal cord and the peripheral properties of muscle. It would then perhaps be surprising that two independent factors should interact so as to give so simple a relationship. If, however, the stretch reflex were primarily dependent upon 'inhibitory feedback' produced by contraction of the muscle, then this particular difficulty would be diminished. If the Golgi tendon organs were the source of such an inhibitory feedback, then the stretch reflex would be regulated to produce some relation between the total tension in soleus and its extension; and this does not seem to be excluded as the normal mode of operation of the stretch reflex. The tension developed in the stretch reflex would then no longer be dominated by variations in the contractile strength of the muscle with its extension, as the efferent discharge would be appropriately adjusted. Indeed, in some cases the efferent discharge might even not increase at all as soleus was extended, and the increase in active tension might depend upon the peripheral properties of soleus, and yet the contraction could remain reflexly controlled.

The slight effect on the tension–extension curve of varying the rate of extension suggests that the discharge of the primary endings of the muscle spindles of soleus may be similarly independent of variations in the rate of extension when this is low, as has been found for some other muscle spindles (Lippold, Redfearn & Vučo, 1959). But the well maintained stretch reflexes sometimes seen in response to a suddenly applied extension suggests that the rate of adaptation of the muscle spindles may have been less than that described by B. H. C. Matthews (1933) from direct recording of their discharges. It is interesting to speculate, therefore, whether the decay of the stretch reflex which might be expected from the adaptation of the muscle spindles was being counteracted by some other factor. The differences between the tension–extension curves obtained on stretching and on releasing soleus probably reflected similar differences in the activity of its muscle spindles during stretch and release. This hysteresis effect shows that the stretch reflex, as studied in soleus, is not strictly reversible on altering the direction of movement, as might otherwise be assumed in considering the nervous control of muscle as a servo-mechanism.

In general, it may be concluded that quantitative analysis of the stretch reflex must await further quantitative studies on the behaviour of various parts of the reflex arc in isolation. Until then the number of unknown variables appears to permit a variety of explanations for a phenomenon whose response often behaves in a surprisingly simple manner.

SUMMARY

1. A study has been made of some of the properties of the tonic (postural) stretch reflex of the soleus muscle of the decerebrate cat (decerebrated by mid-brain section, or by anaemia). Soleus was extended at a slow constant rate (0·5–6 mm/sec) while the tension in it and the extension applied to it were recorded. The experiments provide data on the behaviour of the stretch reflex under a variety of conditions, and raise problems as to the mechanism of its nervous control.

2. When soleus was denervated the passive tension in it increased exponentially with the extension.

3. When the stretch reflex was present its 'active tension' was determined by subtracting the passive tension from the tension actually recorded (total tension). The active tension often increased linearly with the extension, over a wide range of extensions; but when the active tension was low this was no longer so. Sometimes, when the passive tension became large, the rate of increase of active tension with further extension fell. This was probably due to autogenetic inhibition, but in some cases saturation may have occurred in some part of the reflex arc. When 'autogenetic inhibition' occurred the relation between total tension and extension was often more nearly linear than that between active tension and extension. Unfortunately, it has been impossible to state whether, in general, active tension or total tension is better related to the extension by a linear relation. In either case the experimental results were only approximately fitted by a straight line.

4. The preparations used had well maintained stretch reflexes in response to an extension suddenly applied to soleus. When the relation between tension and extension was determined by applying sudden stretches of different lengths, the curve obtained was similar to that obtained by extending soleus slowly and progressively.

5. Varying the rate of extension of soleus fivefold did not alter the tension-extension curve obtained in preparations with large tonic stretch reflexes.

6. When soleus was released at a uniform rate it developed less active tension at any length than it did when it was being extended at the same velocity. A difference was also found between electromyographic records obtained during stretch and during release of soleus. This hysteresis was not affected by altering the velocity at which soleus was released.

7. The tension evoked in soleus by stimulation of its motor nerve was determined over the range of extensions used in the same animals to study the stretch reflex. When the frequency of stimulation was high, so that a fused tetanic contraction was produced at the initial length, extending soleus only slightly increased its active tension. When the frequency of stimulation was low, so that soleus was contracting in an unfused tetanus, extending the muscle greatly increased the active tension developed, and the tetanus became more nearly fused (i.e. extending soleus decreased its 'fusion frequency'). These results were obtained both with maximal and with submaximal activation of soleus.

I should like to thank Professor R. Granit and Dr P. A. Merton for valuable discussion.

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A STUDY OF CERTAIN FACTORS INFLUENCING THE STRETCH REFLEX OF THE DECEREBRATE CAT

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The size of the stretch reflex evoked in a muscle by an extension of fixed magnitude may be modified by influences arising outside the muscle studied. For example, Liddell & Sherrington (1924) showed that the stretch reflex of a muscle may be inhibited in a graded manner by pulling on its antagonist; and Liddel & Olmsted (1929) found that the stretch reflex may be larger than before for about 1 min after eliciting a crossed extensor reflex in the muscle studied. More recently the indirect influence of the γ motoneurones on the stretch reflex has been illustrated by the finding that the size of the stretch reflex may be greatly reduced by selectively paralysing the γ efferent nerve fibres by the application of procaine to the muscle nerve (Matthews & Rushworth, 1957*a*; Matthews, 1958*a*). The present experiments extend previous work by investigating the action of various factors upon the whole tension–extension curve of the stretch reflex, instead of upon the tension developed by an extension of fixed size. This tension–extension curve may be determined by extending the soleus muscle at a slow constant velocity while recording both the tension within it and the extension applied to it (Matthews, 1958*b*, 1959).

METHODS

The present experiments and those described in the preceding paper (Matthews, 1959) were performed as a single series of experiments, upon the same preparations. The Methods section of the preceding paper (pp. 521–4) is thus applicable to the present paper. For the experiments of Part II of the present paper, all the cats were decerebrated by mid-brain section, but for the experiments of Part I cats decerebrated by anaemia were also used. The tension in soleus was recorded by an isometric myograph, and in some experiments this was supplemented by recording the electromyogram of soleus. The stretch reflex was elicited by extending soleus at a constant velocity, which was achieved by moving the myograph by a motor-driven screw thread. The extension applied to soleus at any time during a progressively increasing stretch could be determined from a record of the revolutions of the screw thread. The 'active' or 'reflex' tension elicited by any degree of extension could be determined by subtracting the 'passive tension' (recorded when the stretch reflex was absent) from the 'total tension' (recorded when the stretch reflex was present). This enabled graphs of active tension to be plotted against extension, and in the present paper those obtained under different conditions are compared.

Procaine. In most experiments the nerve to soleus was slowly paralysed by applying procaine solution to it in the manner previously described (Matthews & Rushworth, 1957*a*). A 1·5 × 1·5 cm square of filter paper was soaked in procaine solution (0·15–0·5% procaine hydrochloride in Ringer's solution, pH adjusted to 7·2–7·6), bent into a V and placed over the common nerve to soleus and the lateral head of gastrocnemius, as it lay in a pool of liquid paraffin. With this method of application the procaine paralyses the small gamma motor fibres before the larger alpha motor fibres or the larger afferent fibres (Matthews & Rushworth, 1957*b*, 1958). The duration of the selective paralysis of the γ efferents varied in different experiments from about 3 min to about 20 min. Complete nerve block was procured in times varying from 10 min to over 60 min.

RESULTS

PART I. The effect on the stretch reflex of the progressive paralysis of the nerve to soleus with procaine

When procaine is applied to the nerve to soleus the stretch reflex of soleus is paralysed in two distinct stages, occurring at well separated times (Matthews & Rushworth, 1957*a*; Matthews, 1958*a*). The first stage of paralysis is unaccompanied by any change in the direct motor response of soleus to stimulation of its nerve central to the anaesthetized region, and may be attributed to a selective paralysis of the γ efferent nerve fibres. Figure 1 shows the typical changes produced in the stretch reflex by so paralysing the γ efferents in a cat decerebrated by mid-brain section. The upper points were obtained before applying procaine and indicate the amount of spontaneous variability of the stretch reflex which occurred in this experiment. The lower points were obtained while the nerve to soleus was exposed to procaine solution. During the first 10 min of exposure the stretch reflex became much smaller, but the response to stimulating the nerve central to the anaesthetized region remained unchanged. Over the next 10 min there was little change in the stretch reflex though the direct motor response fell by 20%, showing that the procaine was continuing to anaesthetize the nerve. The two lower curves were therefore obtained with the γ efferents paralysed. When the γ efferents were paralysed the stretch reflex had a threshold and no active tension was developed for extensions below 10 mm, though previously an appreciable active tension had been elicited by an extension of 2 mm. The form of the tension–extension relation was, however, little altered: the curves obtained when the γ efferents were paralysed are approximately linear and their slope differs little from that found when the γ efferents were intact.

The changes which occurred in the stretch reflex when procaine was applied did not occur abruptly, but took about 5 min to develop. This is illustrated in Fig. 2, which consists of superimposed tracings of myographic records obtained in the experiment of Fig. 1. The changes in the stretch reflex consisted essentially of a progressive displacement of the tension–extension curve to the right along the extension axis.

An increase in the threshold of extension for the stretch reflex has been found on paralysing the γ efferents in each of the nine cats decerebrated by mid-brain section in which the action of procaine was studied. In some cases the stretch reflex had a threshold initially and this increased when the γ efferents were completely paralysed; in others slight active tension was developed at the initial length before procaine was applied, and a threshold appeared as the γ efferents were paralysed. In some cases when the γ efferents were completely paralysed the stretch reflex disappeared completely in the range of extension studied; in others it persisted but with a higher threshold than before. The progressive displacement to the right of the curve of active

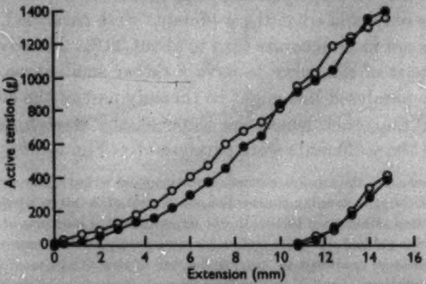

Fig. 1. The effect on the stretch reflex of paralysing the γ efferents by the local application of procaine in a cat decerebrated by mid-brain section. Active tension plotted against extension while soleus was being extended at 1·7 mm/sec. Upper points obtained before applying procaine (○, 5 min before; ●, 2 min before); lower points obtained while the nerve was exposed to procaine (○, 10 min exposure; ●, 20 min exposure) when the γ efferents were believed to be paralysed. (Final passive tension was 430 g).

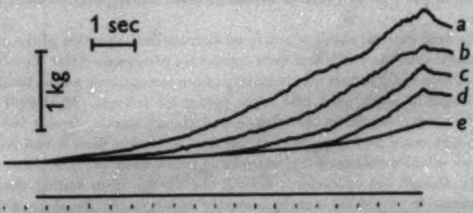

Fig. 2. Superimposed tracings of myographic records to show the progressive changes produced in the stretch reflex by slowly paralysing the γ efferents with procaine. Each record shows the tension produced in soleus by extending it slowly (15 mm at 1·5 mm/sec). The signal lights below apply to all records (maximum error 0·2 mm); upper signal light turned on at beginning of extension, and off when the full extension was applied; lower signal light flashed once for each 0·8 mm increase in the extension. Record *a* was obtained before applying procaine; record *e* was obtained when the stretch reflex was abolished by inhibition; records, *b*, *c*, *d*, were obtained when the nerve had been exposed to procaine for 4·5, 6·5 and 10 min respectively. Same experiment as Fig. 1.

tension against extension could, however, be seen in all experiments as the γ efferents were being paralysed, whether or not any stretch reflex persisted when their paralysis was complete.

In contrast to this regular change of threshold, the slope of the relation between active tension and extension remained relatively unaltered as the γ efferents were paralysed; thus the increase in the threshold appeared to be the principal cause of the change in the stretch reflex. Changes in the slope did occur, but in the whole series of experiments there was no systematic tendency for it to increase or to decrease. The measurement of slope was not particularly accurate, as it was an approximation applicable to a part of the tension-extension curve only, and when the γ efferents were paralysed, measurements were sometimes not more accurate than to about 30 %. Moreover, it was usual for the initial part of the curve to have a rather small slope, and when the γ efferents were paralysed this might be the only part of the curve remaining. Sometimes, on the other hand, the slope of the stretch reflex definitely increased when the γ efferents were paralysed (cf. Fig. 6 *a*, *b*).

These various changes in the tension–extension curve appear to have been due to changes in the 'tonic' properties of the stretch reflex, for it is this component of the stretch reflex which is studied by the present method (Matthews, 1959). In one experiment the response of soleus to different rates of extension was studied when the γ efferents were paralysed. The tension–extension curve was, as usual, found to be unaltered by varying the rate of stretch. In most experiments it was found that the rate of decay of active tension from its peak at the completion of the extension did not change appreciably when the γ efferents were paralysed.

In some experiments it is possible that the increase in the threshold produced by paralysis of the γ efferents depended upon the tendency for soleus then to hang slack at its initial length. The first part of the applied extension took up this slack without extending soleus, and the threshold may have corresponded to the point at which soleus actually began to be extended. This was not, however, the sole cause for the development of a threshold, for often the passive tension became sufficient to pull soleus taut at a smaller extension than that required to elicit the stretch reflex (cf. Fig. 5).

In two of the nine cats the curve of active tension against extension of the stretch reflex was not at all constant when the γ efferents were apparently paralysed. After the nerve to soleus had been stimulated tetanically to test the continuity of the motor fibres, active tension might appear at the initial length of soleus used, and might persist for 1–2 min. Meanwhile the form of the tension–extension curve was flatter than before, and, though starting from a finite tension at zero extension, it might reach no higher level at the final extension than it had reached before the tetanic stimulus. Similar changes were occasionally found when the nerve was unanaesthetized. This variability, though not understood, did not obscure the main finding, that paralysis of the γ efferents increased the threshold more consistently than it altered the slope of the stretch reflex.

Anaemic decerebrates. Of the eight cats decerebrated by anaemia in which the action of procaine was studied, three behaved similarly to those decerebrated by mid-brain section. When the γ efferents were paralysed the threshold of the stretch reflex increased, but the slope of the tension–extension relation did not change greatly. The result of one of these experiments is shown graphically in Fig. 3, and provides a further example of the changes found as the threshold of the stretch reflex altered.

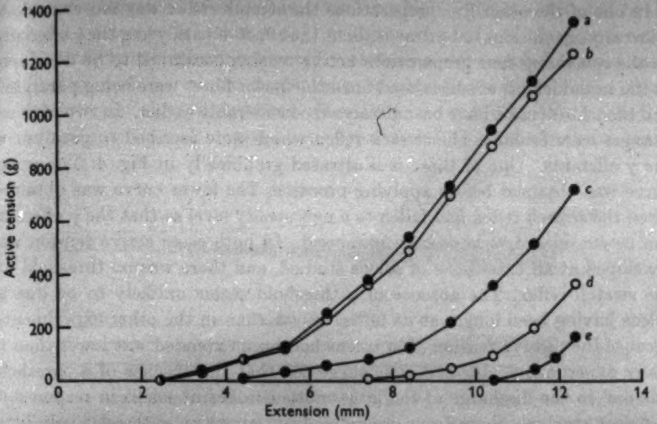

Fig. 3. A graph of the progressive changes in the stretch reflex produced by slowly paralysing the γ efferents with procaine; from an experiment on a cat decerebrated by anaemia. *a*, 1 min before applying procaine; *b*, 90 min later after procaine had been removed and the stretch reflex had returned to its previous size (the nerve was exposed to procaine for 65 min). *c*, *d* and *e* were obtained when the nerve had been exposed to procaine for 6, 9 and 15 min respectively. In *e* the γ efferents were believed to be completely paralysed. In *c* and *d* it is presumed that only some of the γ efferents were paralysed. (Soleus extended 13 mm at 1·6 mm/sec; final passive tension 240 g.)

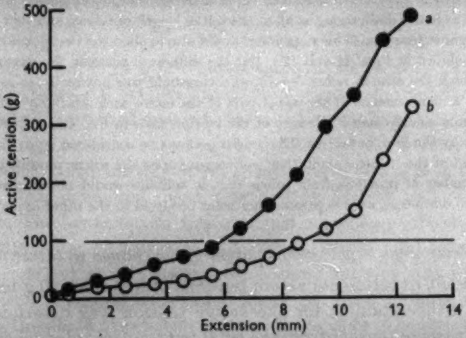

Fig. 4. The effect of paralysing the γ efferents in a preparation (cat decerebrated by anaemia) in which there appeared to be some α motoneurones which were being excited mainly by central pathways with little support from proprioceptors. *a* (●) obtained 4 min before applying procaine; *b* (○) obtained when the nerve had been exposed to procaine for 6 min and the γ efferents were paralysed. In both *a* and *b* active tension was developed at all extensions studied. (Soleus extended at 1·7 mm/sec; final passive tension for 13 mm extension was 210 g. The horizontal line is drawn at the arbitrary level of 100 g in order to emphasize the two limbs of the curves.)

In one of the other five preparations the stretch reflex was too variable to allow any conclusions to be drawn about the effect of paralysing the γ efferents. In the remaining four preparations active tension continued to be developed at the initial length of soleus used until the motor fibres were being paralysed, and the γ fibres must have been paralysed considerably earlier. In two of these, changes were found in the stretch reflex which were ascribed to paralysis of the γ efferents. One of these is illustrated graphically in Fig. 4. The upper curve was obtained before applying procaine. The lower curve was obtained when the stretch reflex had fallen to a new steady level so that the γ efferents can be considered to have been paralysed. In both cases active tension was developed at all extensions of soleus studied, and there was no threshold for the stretch reflex. The absence of a threshold seems unlikely to be due to soleus having been longer at its initial length than in the other experiments, because the passive tension after soleus had been extended was lower than in many experiments. Instead, it is suggested that the absence of a threshold was due to the discharge of the large motoneurones of soleus in response to impulses reaching them from purely central pathways, with relatively little support from impulses from the soleus proprioceptors; for anaemic decerebrates may retain extensor rigidity after the dorsal roots have been cut (Pollock & Davis, 1930, 1931).

Partial support of this idea is afforded by the flatness of the initial part of the tension–extension curves of Fig. 4. For the first 66 mm of extension the average slope was only 15 g/mm for *a* and 7 g/mm for *b*, whereas for the last 2 mm of extension it was 70 g/mm for both, and this is a more usual figure. One interpretation of this small value of the slope at low extension is that some of the muscle spindles were not discharging at all at the initial length of soleus, and that these were only recruited by larger extensions. (This suggestion would also explain the two slopes of the facilitated stretch reflexes shown in Figs. 11 and 12.) But the difference between the experiment of Fig. 4 and those in which the stretch reflex developed a threshold was not really so great, for in both cases there was a displacement of the upper part of the curve with relatively little change in its slope. This is more clearly seen if the zero of the tension scale in Fig. 4 is displaced upwards by 100 g, as shown by the horizontal line. This might perhaps be considered equivalent to removing a certain amount of the 'central excitation' converging upon the soleus motoneurones, so that a considerable number of impulses from soleus muscle spindles would be required to excite the motoneurones to discharge, as was presumably what occurred in the other experiments.

Electromyographic confirmation of the change in threshold

In the present experiments active tensions of less than 20 g have not been measurable with certainty, for the active tension was determined by subtracting the passive tension from the total tension (recorded when the stretch reflex was present), and neither was measured more accurately than to about 10 g. Thus at the 'threshold' of extension for the stretch reflex as determined by myography several motoneurones might already have been excited to discharge. In thirteen of the experiments, therefore, electromyography has been used to supplement myography and to confirm that the stretch reflex may have a threshold of extension. One recording lead was connected to the insulated tendon hook and the other placed on the belly of soleus or near its origin. In eight of these experiments the gastrocnemius muscle was completely denervated, and there is no doubt that the potentials recorded arose solely from soleus, for they disappeared when its nerve was cut. This method of recording probably picked up the activity of most of the motor units in soleus; and often the discharge of a few motor units could be detected at a period during a slow extension when no appreciable active tension had been developed. A result from an experiment on a cat with a rather small stretch reflex is shown in Fig. 5. The upper record was obtained before applying procaine. There was no detectable electrical activity at the initial length of soleus in this case, but it appeared when soleus had been extended by 3·5 mm and then increased progressively. The lower record was obtained with the γ efferents paralysed, when only 80 g of active tension was developed at the final extension. When soleus was stretched at this stage, no electrical activity was recorded until soleus had been extended by 11 mm. Thus, paralysing the γ efferents had increased the threshold of extension for the appearance of electrical activity by 7·5 mm. Similar results have been obtained in most other experiments.

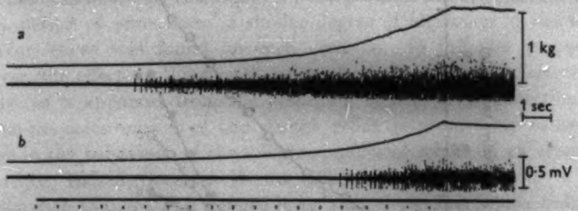

Fig. 5. An increase of the threshold of the stretch reflex shown by recording the electrical activity of soleus (belly–tendon leads). Records of the tension in soleus (above) and its electrical activity (below) obtained on slowly extending it *a*, when the nerve was intact, *b* when the γ efferents were paralysed by procaine. The signal lights monitoring the extension are applicable to both records; upper light turned on at beginning of extension (end of extension not signalled); lower light flashed once for each 0·8 mm increase in the extension. When the γ efferents were paralysed no electrical activity was recorded until soleus had been extended by 11 mm, whereas previously it had begun after 3·5 mm of extension. (Extension 15 mm, at 1·1 mm/sec; stretch reflex was rather small when γ efferents were paralysed and most of tension then recorded was passive tension, the final level of which was 500 g at full extension. The electrical record was cut off abruptly at the bottom of the record because it then fell out of the field of view of the camera.)

The increase in threshold as judged by the electrical discharge was not always identical with that found from the graphs of active tension against extension. In the experiment of Fig. 5 the shift of threshold found by plotting the active tension against extension was 6 mm. The difference between the two methods lies in their sensitivity, for the electrical record may show the discharge of the first motor unit to be excited by stretch of soleus, while the active tension does not increase appreciably until several motor units are active. The electrical recording sometimes also showed that, when the stretch reflex had apparently developed a threshold as shown by the mechanical record, there might be one or more motor units still discharging at the initial length of soleus. In most cases, however, the electromyographic experiments have confirmed that when the γ efferents were paralysed a larger extension of soleus than before was required to elicit a stretch reflex.

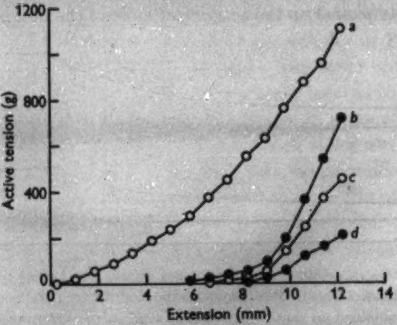

Fig. 6. The effect on the stretch reflex of soleus of progressively paralysing its large nerve fibres. *a*, obtained 2 min before applying procaine; *b*, *c* and *d* obtained when the nerve had been exposed to procaine for 11, 15 and 16 min respectively. The difference between *a* and *b* is attributed to paralysis of the γ efferents. In this experiment the slope of the tension–extension relation increased when the γ efferents were paralysed. The differences between *b*, *c* and *d* are attributed to progressive paralysis of the large nerve fibres of soleus (probably afferent as well as efferent) and consist primarily of changes in the slope of the tension–extension relation. (Extension 13 mm at 1·2 mm/sec; final passive tension 230 g.)

Paralysis of large fibres

The changes produced in the tension–extension curve by paralysis of the larger fibres supplying soleus could be clearly differentiated from those produced by paralysis of the γ efferents. The principal change was a consistent decrease in the slope of the tension–extension relation. It could only be studied in experiments in which an appreciable stretch reflex persisted after the γ efferents were paralysed, but it has been seen in 10 applications of procaine in six preparations (four anaemic decerebrates, two classical decerebrates). One example is illustrated in Fig. 6, obtained from a cat decerebrated by mid-brain section. Curve *b* was obtained when the γ efferents were paralysed, and at this time the direct motor response, elicited by stimulation of the nerve at 90/sec central to the anaesthetized region, had been reduced to 90 % of its original value. Curves *c* and *d* were obtained as the motor fibres were being progressively paralysed, and the direct motor responses had been reduced to 50 and 30 % respectively of their original values. Electromyography has confirmed that the threshold of the reflex usually alters little at this stage. It is not certain whether this change of slope is due primarily to a paralysis of the afferent pathway or of the efferent pathway of the stretch reflex (or both) and both would be expected to be paralysed at about the same time (Matthews & Rushworth, 1957 *b*).

Comparison of the activity of the γ motoneurones in different preparations

In any one preparation the influence of the γ motoneurones on the stretch reflex has been relatively constant, for when procaine was applied several times over a period of some hours a similar degree of alteration in the tension–extension curve was found on each occasion. In different preparations, however, the effect on the stretch reflex of paralysing the γ efferents varied greatly, as is apparent from the preceding figures. This alteration of the tension–extension curves of the stretch reflex is presumably caused by a change in the behaviour of the muscle spindles of soleus on paralysis of the γ efferents. Eldred, Granit & Merton (1953) introduced the term 'γ bias' to describe such changes, and defined it as 'that shortening which reduces the rate of the biased spindle to that of the unbiased spindle at the original length'. If it be assumed that a given number of spindle impulses reflexly causes a constant active tension to be developed in soleus, the displacement found in the tension–extension curves of the stretch reflex may be interpreted as giving a composite figure for the γ bias of the spindles of soleus. When the tension–extension curves are not parallel before and after paralysing the γ efferents, the γ bias is not constant, but neither did Eldred *et al.* (1953) find it to be so for a single spindle.

For comparison of the different experiments the 'γ bias' has been measured at the arbitrary level of 100 g active tension. The determination was usually accurate only to 1–2 mm, because of the variability of the stretch reflex. In five of the cats decerebrated by mid-brain section 100 g or more of active tension persisted at the final extension when the γ efferents were paralysed, and their γ bias ranged from 5 to 10 mm (mean, 8·4 mm). In the four others studied not so much active tension remained, or none at all, and only a minimum value can be given to the γ bias. This ranged from 4 to 7 mm (mean, 6·1 mm.). In the eight cats decerebrated by anaemia which were studied, four had biases ranging from 1 to 4 mm, which is lower than any found in the cats decerebrated by mid-brain section. One had rather little γ bias, but the stretch reflex was too variable, both when the γ efferents were paralysed and when they were intact, to assign to it a definite value. The remaining three anaemic decerebrates had γ biases which were comparable to those of the ordinary decerebrates (7, 7 and over 9 mm). The greater variability of the cats decerebrated by anaemia is perhaps not surprising, as the amount and the location of brain destroyed must depend partly upon the distribution of the smaller blood vessels, and partly upon the extent of development of a collateral circulation, both of which may well vary from preparation to preparation. The difference between the two preparations is in accordance with that described by Granit, Holmgren & Merton (1955), who found that 'in general the muscle spindles were biased less in anaemic preparations than in those decerebrated by inter- or pre-collicular section'; though in the present experiments the level of section decerebration has usually been lower.

PART II. The influence of certain other reflexes on the stretch reflex

The effect on the stretch reflex of exciting the soleus motoneurones

The soleus motoneurones could be readily excited, independently of the stretch reflex, by stimulating the contralateral saphenous nerve. Figure 7 shows the effect of combining the stretch reflex with such a crossed extensor reflex. The two upper curves were obtained during the crossed extensor reflex (*b*), and immediately afterwards when the stretch reflex was facilitated (*c*). The two lower curves show the size of the unfacilitated stretch reflex. For extensions of up to 9 mm the stretched reflex and the crossed extensor reflex together produced a greater tension than the sum of their individual effects, that is to say they produced mutual facilitation. For extensions of from 9 to 15 mm, the crossed extensor reflex produced a constant additional tension in soleus in spite of a threefold increase in the size of the stretch reflex. The 'facilitatory after-effect' of the crossed extensor reflex produced a similar, but smaller, effect on the stretch reflex. Thus in both cases the slope of the tension–extension curve of the stretch reflex was little altered by facilitating the soleus motoneurones. This effect was readily repeatable in the preparation of Fig. 7,

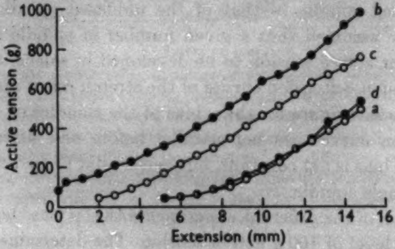

Fig. 7. The effect on the stretch reflex of simultaneously eliciting a crossed extensor reflex in soleus. Curve *a*, stretch reflex evoked by extending soleus (15 mm at 2·6 mm/sec) in the absence of other stimulation; curve *b* was obtained 20 sec later by extending soleus during the plateau of the crossed extensor reflex produced by stimulating the contralateral saphenous nerve at 90/sec. Curve *c* was obtained 15 sec and curve *d* 2 min after stopping the stimulation. The crossed extensor reflex and its 'facilitatory after-effect' added an approximately constant tension to the stretch reflex. (Slight active tension was present at the initial length in all cases, but points showing less than 40 g active tension have not been plotted. Final passive tension was 570 g.)

and a similar change in the tension–extension curve has been seen in four other experiments. But the curve obtained during stimulation of the saphenous nerve was not always smooth, especially when the resulting crossed extensor reflex was large, and the reflex was often 'clonic' rather than 'tonic'. The curves obtained immediately after stimulating the saphenous nerve were usually more regular, and it is with these curves that the most satisfactory comparisons with the normal curve have been possible.

A more constant degree of excitation of the soleus motoneurones could be produced by stimulating the central end of the cut nerve to the medial head of gastrocnemius of the same side (cf. Alvord & Fuortes, 1953). After 1–2 sec of stimulation with weak repetitive shocks a very constant tension was usually maintained as long as stimulation was continued (30–60 sec).

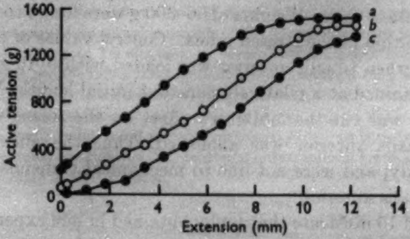

Fig. 8. The effect on the stretch reflex of simultaneous stimulation of the central end of the cut nerve to the medial head of gastrocnemius. Curve *c* was obtained without stimulation of GM nerve. Curves *a* and *b* were obtained while the GM nerve was being stimulated at 90/sec (*a*) and at 55/sec (*b*). The tops of the curves were probably limited by 'saturation' of some part of the reflex arc, otherwise result similar to that of Fig. 7 for the crossed extensor reflex (the active tension in *a* increased to 85% of that produced by stimulation of the motor fibres of soleus at 90/sec). (Soleus extended 13 mm at 1·3 mm/sec, final passive tension 200 g.)

Figure 8 illustrates such an experiment. In *b* the additional reflex, superimposed on the stretch reflex, produced an approximately constant increase in tension over an appreciable range of extensions, but in *a* the slope of the tension–extension curve also altered. This experiment has been illustrated because of the interest of the flattening of the curves seen at tensions above 1300 g. This was probably due to saturation of some part of the reflex arc, the possibility of which has been considered earlier (Matthews, 1959). (An active tension of about 1500 g proved to be a very constant ceiling for the stretch reflex in this experiment. Moreover, the maximal tetanic tension developed by stimulating the soleus motor fibres at 90/sec was 1800 g when soleus was extended.) The effect of stimulating the nerve to the medial head of gastrocnemius was also studied in three other preparations, but in these the effect was smaller (similar to *b* of Fig. 8). In all these the effect of exciting the soleus motoneurones while a stretch reflex was being elicited has been to add a relatively constant amount of tension to the stretch reflex.

The effect on the stretch reflex of inhibiting the soleus motoneurones

A partial inhibition of the stretch reflex of soleus was produced by pulling on the tendon of its antagonist, tibialis anterior. For these experiments tibialis anterior was, of course, left innervated. (Instead of cutting the whole lateral popliteal nerve in the thigh, as was otherwise usual, only its musculocutaneous branch in the leg was cut.) The tendon of tibialis anterior was pulled upon by connecting it by a string running over a light pulley to a weight hanging vertically downwards. Observations were made when tibialis anterior had been loaded for more than 1 min, when it is probable that its length had settled to a constant value. Weights of 50–300 g were found to produce suitable degress of inhibition of the stretch reflex. Control values of the stretch reflex were obtained when tibialis anterior was loaded with 5–10 g in order that it should be maintained at a relatively constant initial length. When the lateral popliteal nerve was cut the inhibitory effect on the soleus motoneurones of pulling on tibialis anterior was abolished. Thus the effects studied were mediated reflexly, and were not due to mechanical transmission to soleus of the pull on tibialis anterior.

Figures 9 and 10 illustrate the results obtained in one experiment. Figure 9 consists of superimposed tracings of the myographic records obtained when tibialis anterior was loaded with 5 g (*a*) and with 100 g (*b*). Figure 10 shows graphs of active tension against extension for the normal stretch reflex (*a*, *b*), and for the partially inhibited stretch reflex (*c*). In both figures it can be seen that, at extensions at which the stretch reflex was well developed, loading tibialis anterior decreased the active tension by an amount which was approximately constant. The slope of the upper part of the tension–extension curve was not altered. The effect on soleus of loading tibialis anterior was examined several times in this preparation, with results similar to those illustrated. Smaller loads on tibialis anterior produced a smaller effect, and larger loads a larger effect; but this has not been investigated quantitatively. Similar results have been obtained in two other preparations. In these, however, there appeared to be a slight decrease (25–30 %) in the slope of the relation between active tension and extension when tibialis anterior was loaded. In the range of extensions studied, however, the principal change in the stretch reflex could be ascribed to displacement of the curve rather than to this change of its slope. Another preparation gave confirmatory results, but in this cat the stretch reflex was spontaneously variable.

In three other experiments the stretch reflex of soleus was partly inhibited by stimulating electrically the central end of the cut ipsilateral lateral popliteal nerve. Weak shocks were required, so as to avoid inhibiting the stretch reflex completely, and it was found difficult to produce a constant effect. The lateral popliteal nerve contains several kinds of fibres whose stimulation would be likely to inhibit the stretch reflex of soleus, and it is not known which of these were being excited. In these experiments again, however, the inhibition decreased the stretch reflex by an approximately constant amount in the range of extensions in which it was normally well developed.

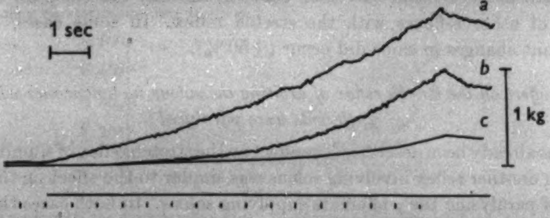

Fig. 9. The effect on the stretch reflex of soleus of pulling on its antagonist, tibialis anterior. Superimposed tracings of myographic records of the tension developed in soleus when it was slowly extended (12 mm at 1·3 mm/sec). *a*, control, stretch reflex normal (5 g weight on tibialis anterior). *b*, stretch reflex partially inhibited by hanging a 100 g weight on the tendon of the ipsilateral tibialis anterior muscle. *c*, passive tension produced by the extension (stretch reflex inhibited by stimulation of the hamstring nerve). Signal lights below monitor the extension and are applicable to all records (maximum error less than 0·2 mm); upper light turned on at beginning of extension (full extension not signalled); lower light flashed once for each 0·8 mm increase in the extension.

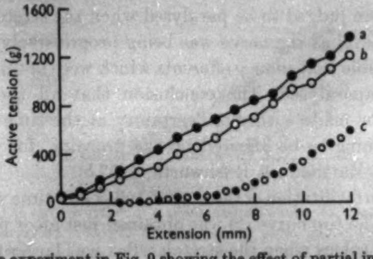

Fig. 10. A graph of the experiment in Fig. 9 showing the effect of partial inhibition of the stretch reflex. *c*, stretch reflex partially inhibited by loading tibialis anterior with 100 g, results of two successive stretches plotted (●, ○). *a* and *b* control stretch reflexes obtained before (*b*) and after (*a*) obtaining curve *c*. The inhibition decreased the active tension in soleus by an approximately constant amount in the range of extensions in which an appreciable stretch reflex persisted.

Spontaneous variations in the stretch reflex. As has already been discussed in the preceding paper, minor variations in the stretch reflex are common (cf. Fig. 6, Matthews, 1959). It did not appear useful to attempt to analyse them into changes of slope or changes of 'threshold' of the tension–extension curve. Sometimes large changes in the stretch reflex occurred spontaneously; they were often associated with changes in the amount of extensor rigidity in the other limbs of the preparation. Usually these changes were due to a displacement of the tension–extension curve in the way described for the interaction of other reflexes with the stretch reflex. In some cases, however, significant changes in slope did occur (±50%).

The effect on the stretch reflex of exciting the soleus motoneurones when the γ efferents were paralysed

As has already been described, the effect on the stretch reflex of simultaneously eliciting another reflex involving soleus was similar to the effect on the stretch reflex of paralysing the γ efferents supplying soleus. In both cases the change in the stretch reflex could be described rather as a displacement of the tension–extension curve along one or other axis than as a change of its slope. It is probable that part of the effect produced by the other reflexes was mediated by a change in the level of discharge of the γ motoneurones of soleus. It seems unlikely, however, that the effects of the reflexes were mediated solely through the γ motoneurones; and a few results have been obtained showing that small changes may still be produced reflexly in the tension–extension curve of the stretch reflex after the γ efferents have been paralysed by procaine. As before, the γ efferents were judged to be paralysed when the stretch reflex had fallen to a new steady level as the nerve was being progressively anaesthetized. In this case it is possible that some γ efferents which were not firing spontaneously still remained unparalysed. The conclusion that all the γ efferents were paralysed could be made with more certainty at the time when the α motor fibres were beginning to be affected by the procaine, for the γ efferents are paralysed earlier (Matthews & Rushworth, 1957 *b*).

Figure 11 shows facilitation of the stretch reflex at a time when the γ efferents were paralysed. The top curve (*b*) was obtained just after producing a crossed extensor reflex in soleus when the stretch reflex was larger than that obtained earlier (*a*) or later (*c*). At this stage of narcosis the motor fibres were unaffected by the procaine, but a similar facilitation was produced 5 min later, when the tension developed in soleus on stimulation of its nerve at 90/sec was decreasing. The differences between the curves of Fig. 11 again appear to be due to a displacement of the curve rather than to any change of its slopes, although none of the curves is fitted by a straight line more than half its extent. Certainly there appears to be little difference of slope between the upper parts of curves *b* and *c*. These changes must have been mediated through the α motoneurones.

Similar changes were obtained in the same preparation during another application of procaine and in three other preparations (during one application of procaine for each). In one, the 'facilitatory after-effect' on the stretch reflex of the crossed extensor reflex was studied. In another, the nerve to the medial head of gastrocnemius was stimulated while soleus was being extended. This was also done in the third preparation, and although the results obtained during the stimulation were anomalous, a shift was seen during a period of 'facilitatory after-effect' occurring after one period of stimulation. The changes seen in these experiments were slightly smaller than those shown in Fig. 11. Though the curve was displaced, it was uncertain whether or not its slope changed, for appreciable active tension was only present over a small range of extensions, even when the soleus motoneurones were facilitated.

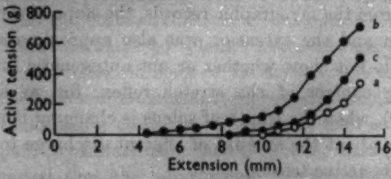

Fig. 11. The effect on the stretch reflex of the 'facilitatory after-effect' of the crossed extensor reflex at a time when the γ efferents were paralysed. The curves were obtained in the order *a*, *b*, *c* after the nerve to soleus had been exposed to procaine for 9, 10 and 11 min respectively. Between obtaining *a* and *b* the contralateral saphenous nerve was stimulated, giving rise to a crossed extensor reflex in soleus. This left some 'facilitatory after-effect' so that the stretch reflex became temporarily larger in *b*. This 'after-effect' was only temporary, as is shown by the decrease of the stretch reflex seen in *c*. The difference between the curves must have depended on changes in the 'excitability' of the α motoneurones, and cannot have been mediated through the γ motoneurones. (Extension at 1·7 mm/sec; final passive tension 280 g.)

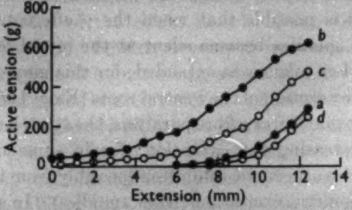

Fig. 12. The effect on the stretch reflex of a spontaneous increase in 'tone' at a time when the γ efferents were paralysed. Records obtained in the order *a*, *b*, *c*, *d* (5·5, 6·5, 7 and 8 min exposure of the nerve to procaine; the γ fibres were paralysed after 3 min exposure, and the large motor fibres were beginning to be paralysed after 8 min). Between *a* and *b* there was a sudden spontaneous increase in the stretch reflex, which then gradually returned to the previous level to which it had fallen when the γ efferents were paralysed. (Extension at 1·9 mm/sec; passive tension at final extension 390 g.)

A more striking result was obtained by chance in a preparation in which the 'tone' increased spontaneously at a time when the γ efferents were paralysed. It is illustrated in Fig. 12. The two lower curves (*a*, *d*) show the equilibrium size of the stretch reflex with the γ efferents paralysed. The two upper curves were obtained between determining the two lower curves, and they show the spontaneously facilitated stretch reflex. In this isolated occurrence there was a striking displacement of the curve with little change of its slope.

DISCUSSION

The present experiments show that under a wide variety of conditions changes in the size of the stretch reflex depend upon changes in the threshold of the reflex rather than upon changes in the slope of its tension–extension relation. The results have been described in terms of the active tension in soleus, but, as may be seen from the myographic records, the slope of the relation between the total tension and the extension was also approximately constant. The present results do not show whether or not autogenetic inhibition is of importance in the control of the stretch reflex; for, as already explained (Matthews, 1959), when the length of soleus is changing it is very difficult to draw conclusions about the amount of efferent discharge to the muscle from a knowledge of its active tension.

The threshold of the stretch reflex was presumably overstepped when there were sufficient excitatory impulses converging on the soleus motoneurones, whether these impulses were derived from the muscle spindles of soleus or from other sources. Paralysis of the γ efferents would be expected to increase the threshold, for it would reduce the number of impulses elicited from the muscle spindles by any particular extension of soleus. When active tension persisted at the initial length of soleus with the γ efferents paralysed, it may have depended primarily upon afferent impulses from sources other than soleus. Indeed, it is possible that when the γ efferents were paralysed, a number of muscle spindles became silent at the initial length of soleus, and were only recruited as soleus was extended; for this may occur after inactivation of the γ fibres by section of the ventral roots (Eldred *et al.* 1953, figs, 6, 15). It appears unlikely that, after γ fibre paralysis, the stretch reflex can invariably be restored by increasing the extension; for in some cases this might be expected to increase autogenetic inhibition (possibly from tendon organs) more rapidly than autogenetic excitation (from spindles). In most of the present experiments, however, the stretch reflex was restored when the extension had been increased to near the physiological limit (as judged by the value of the passive tension).

It is tempting to relate the slope of the tension–extension curve to the slope of the relation between the extension and the frequency of discharge of the primary endings of the muscle spindles (spindle sensitivity). The constancy of the slope of the stretch reflex under different conditions might then be taken to suggest that the spindle sensitivity was also remaining constant. The sensitivity of the spindles of the cat's soleus, however, tends to increase with increasing γ motoneurone discharge, though the change in sensitivity may be small in relation to the alteration in the γ bias (Eldred *et al.* 1953). Perhaps any consistent change in the slope of the stretch reflex resulting from paralysis of the γ efferents was masked by a concurrent paralysis of the Group II afferents. These are probably inhibitory for the stretch reflex, and their paralysis might be expected to increase the slope of the tension–extension curve, as was occasionally found (Fig. 6).

The experiments as a whole suggest that reflexes influencing either the α or the γ motoneurones may change the active tension of the stretch reflex by an amount which remains approximately constant as the length of the muscle is altered. This is of interest because a constant active tension may not correspond to a constant efferent discharge to soleus. Moreover, the constancy of the effect of the additional reflex, in spite of variation in the size of the stretch reflex, shows that occlusion or facilitation (expressed in terms of tension) is not inevitable when two reflexes converge on the same final common path. The present result apparently differs from that of Koella, Nakao, Evans & Wada (1956), who suggested that the labyrinthine reflexes sum with the stretch reflex in a 'multiplicative manner'. Their experiments differed, however, in many respects from the present experiments and were performed on preparations given eserine, which affects the behaviour of the Renshaw cells (Eccles, Fatt & Koketsu, 1954) and may have altered the behaviour of the stretch reflex.

SUMMARY

1. A study has been made of the interaction of various factors affecting the size of the stretch reflex of the soleus muscle of the decerebrate cat. The relation between the active tension developed in soleus and the extension applied to it was determined by extending soleus at a slow constant velocity. The changes produced in the stretch reflex are of interest when attempting to understand the co-ordination of different reflexes affecting a single muscle.

2. The effect of the activity of the γ motoneurones was investigated by selectively paralysing their nerve fibres by the application of procaine solution to the nerve to soleus. When the γ efferents were paralysed the active tension usually disappeared at small extensions of soleus, but reappeared when soleus was extended further; that is, the stretch reflex had altered its threshold of extension. In these cases the slope of the linear portion of the relation between active tension and extension was often relatively little altered by paralysis of the γ efferents, and no consistent change in slope has been found. In other cases (especially in cats decerebrated by anaemia) active tension still persisted at the initial length of soleus, and the slope of the initial part of the curve of tension against extension might be small. At larger extensions, however, the slope of the tension–extension relation was similar to that obtained before applying procaine. Paralysis of the γ efferents produced on average larger changes in the stretch reflexes of cats decerebrated by midbrain section than in those of cats decerebrated by anaemia.

3. In contrast to the effect of paralysing small fibres, paralysis of the large

fibres supplying soleus consistently decreased the slope of the tension–extension relation.

4. The effect on the stretch reflex was studied of simultaneously eliciting another reflex influencing the soleus motoneurones. The active tension in soleus increased or decreased by an amount which remained approximately constant during progressive extension in the range of extensions in which the stretch reflex was well developed; thus, there was little change in the slope of the relation between active tension and the extension of soleus. Excitatory effects were produced either by stimulating the contralateral saphenous nerve, or by stimulating the ipsilateral nerve to the medial head of gastrocnemius. Inhibitory effects were produced either by hanging a weight on the ipsilateral tibialis anterior muscle, or by stimulating the lateral popliteal nerve of the same side. Excitatory effects could still be demonstrated when the γ efferents were paralysed by procaine.

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SUPRASPINAL CONTROL OF INTERNEURONES MEDIATING SPINAL REFLEXES

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A number of investigations have been devoted to the functional organization of synaptic actions by impulses in muscle afferents. The excitatory and inhibitory actions by impulses in I a afferents (spindle afferents with annulospiral endings) have been found to be distributed to the different motor nuclei according to an intricate pattern suggesting that the I a system has the double function of subserving both postural reflexes and co-ordination of movements (Liddell & Sherrington, 1924; Lloyd, 1946*b*; Laporte & Lloyd, 1952; Eccles, Eccles & Lundberg, 1957*b*; Eccles & Lundberg, 1958*c*).

The synaptic actions by impulses in I b afferents (i.e. from Golgi tendon organs) have also been investigated in detail and found to have a characteristic distribution (Laporte & Lloyd, 1952; Eccles, Eccles & Lundberg, 1957*c*). In part this pattern of action suggests that by autogenetic inhibition the I b system provides protection against excessive tension (cf. Granit, 1950; Granit & Ström, 1951). Widespread effects from extensor muscles and in particular from flexor digitorum longus (Eccles *et al.* 1957*c*) indicate that the I b system may also be of importance in terminating the extension phase and initiating the flexion phase of stepping (Eccles & Lundberg, 1958*a*).

The reflex action of Group II afferents (12–4μ) from the flower-spray endings on muscle spindles (Hunt, 1954), and also of the still smaller Group III afferents ($< 4\mu$) are those of the general flexion reflex (Lloyd, 1943; Brock, Eccles & Rall, 1951; Laporte & Lloyd, 1952; Eccles & Lundberg, 1959).

The actions by impulses in Group II fibres are of particular interest. Group II fibres are activated by a slight stretch of the muscle. The threshold for excitation is only slightly higher than for the I a fibres (i.e. from annulospiral endings) (Hunt, 1954). Likewise both type of muscle-spindle afferents may be discharged by impulses in γ-efferents (Hunt, 1954). Thus there are two systems of afferents from muscle spindles which are activated similarly and yet with different central connexions to motoneurones. From this fact there arose the following problem: The pattern of Ia action is apparently well adapted to subserve locomotion, yet it is difficult to see how these connexions assert themselves in the reflex control of movement if superimposed on the very large non-specific actions of Group II impulses.

One possible answer is that the reflex actions in the spinal preparation are not characteristic of those in the non-spinal animal. Originally it was believed that in the cat spinal transection was followed by depression of all reflexes in the regions posterior to the transection (Sherrington, 1906). Later Sherrington & Sowton (1915) and Forbes, Cobb & Cattell (1923) found that spinal transection actually increased the flexion reflex evoked by single stimuli. It has also been shown that inhibitory reflex actions may be increased considerably after spinal transection. The increased susceptibility of the knee jerk to inhibitory stimuli (Ballif, Fulton & Liddell, 1925; Fulton, Liddell & Rioch, 1930*a*, *b*; Liddell, 1934, 1936) indicates that inhibitory reflex actions may also be increased after spinal section. Fulton (1926) suggested that the release was due to disappearance of inhibitory action on the interneurones mediating the reflexes, and decisive experimental evidence in support of this suggestion was given by Job (1953). A control of interneurones in the dorsal horn from higher centres was postulated by Austin (1952), by Lindblom & Ottosson (1955) and by Kleyntjens, Koizumi & Brooks (1955), who found that components of the cord dorsum potential and polysynaptic reflexes were decreased on stimulation of the reticular formation. Hagbarth & Kerr (1954) studied the control of transmission in ascending tracts.

In the present series of experiments the synaptic actions exerted on motoneurones by the individual systems of muscle afferents have been compared in decerebrate and spinal cats (cf. Job, 1953). It will be demonstrated that supraspinal centres exert a tonic inhibitory action on interneurones that relay impulses in Group Ib, II and III afferents, whereas no evidence for such a control has been obtained for interneurones mediating Ia inhibitory actions. A preliminary report of some of the results has been published (Eccles & Lundberg, 1958*a*).

METHODS

These experiments were done on cats decerebrated by intercollicular section, using pentothal or ether as the anaesthetic. The decerebration was performed when the animal had been mounted in the frame and all other dissection had been completed. In most experiments transection of the spinal cord was made in the first lumbar segment after local injection of cocaine. Special reference will be made to the experiment in which the cord was divided more rostrally. Since the anaesthetics employed were all short-acting the results may be considered to be from animals virtually unanaesthetized.

In most of the experiments the synaptic actions were measured by their action on testing monosynaptic reflexes recorded in the ventral roots (Renshaw, 1940; Lloyd, 1941, 1946*a*). In some experiments intracellular records were taken from motoneurones, the excitatory and inhibitory post-synaptic potentials (EPSP and IPSP) giving a direct measure of the respective synaptic actions (Eccles, Eccles & Lundberg, 1957*a*, *b*, *c*; Eccles & Lundberg, 1959). In order to investigate the synaptic actions of the various groups of muscle afferents, volleys were evoked by stimuli of different strengths, which were expressed in multiples of the threshold strength.

When recording monosynaptic reflexes two volleys were often used to evoke the monosynaptic reflex used for testing. The strength of the first volley was subliminal for reflex excitation, and preceded the second stronger volley by an interval that gave maximal facilitation of the monosynaptic reflex evoked by the second volley. With this technique larger and more stable reflexes can be elicited. An important advantage was that the size of the monosynaptic reflex could be altered simply by varying the strength of the first shock. Thus the monosynaptic reflex could be adjusted to the same size after spinal transection. Furthermore, with the double-volley technique it was often possible to evoke monosynaptic reflexes which could not be potentiated post-tetanically and which therefore may have comprised the whole motoneurone pool (Beswick & Evanson, 1955). However, in order to ensure an adequate subliminal fringe, the test reflexes were usually about one third of maximal size.

For experiments on monosynaptic reflexes, the time-consuming procedure was employed of obtaining interaction curves at 4–6 different conditioning strengths. At each strength 10–15 different intervals were selected and 10–20 superimposed records at a frequency of 1/sec were taken at each interval. The mean of these records was plotted as a percentage of the mean control reflex, with time as the abscissa.

When the conditioning afferent volley displayed the separation into Ia and Ib components, it was possible to differentiate between the actions of Ia and Ib impulses (cf. Bradley & Eccles, 1953; Laporte & Bessou, 1957; Eccles *et al.* 1957*a*). This procedure was important in those cases in which the conditioning volley gave a monosynaptic reflex which would cause recurrent (Renshaw) inhibition (cf. Eccles, Fatt & Koketsu, 1954). When there was no separation into Ia and Ib components, the stimulus setting of the conditioning volley was adjusted so that it was just maximal for the monosynaptic reflex and the effects of this volley were compared with those obtained when the conditioning strength was maximal for Group I. Any difference in action can then be ascribed to Ib impulses. This method gives significant results because, even in the absence of separation in Ia and Ib components by conduction velocity, there is usually a considerable difference in threshold between these two groups of afferents (cf. Eccles *et al.* 1957*a*). Renshaw inhibition secondary to the discharge of impulses by motoneurones presents a complication in an analysis of the present type. For interpretation of the present results it is of importance to know that there is no release of Renshaw inhibitory action after spinal transection (Holmqvist & Lundberg, unpublished).

Discriminative tests were also necessary in order to differentiate between actions caused by high-threshold Ib fibres and low-threshold Group II fibres. For this purpose the actions were examined at strengths just maximal and then slightly supramaximal for Group I, and, when no increase of action occurred, the effects could be ascribed to impulses in Group Ib fibres. Even in unanaesthetized animals it was a constant finding that despite the overlap in threshold with Ib afferents, the actions by Group II afferents did not appear until the stimulus strength was raised markedly above the strengths needed for the maximal activation of Group I. Presumably a considerable summation is needed for activation of the interneurones mediating Group II actions. There is also a differentiation of effects caused by Group II and III afferents (Eccles & Lundberg, 1959).

RESULTS

Flexor nuclei

Action of impulses in muscle afferents. The curves in Fig. 1 illustrate the effect produced by conditioning volleys in the plantaris nerve upon the monosynaptic reflex from the posterior biceps-semitendinosus (BSt) nerve. At all strengths there was no significant facilitatory action in the decerebrate state (circles). After complete section of the spinal cord at L1 the facilitatory actions characteristic of the spinal animal appeared. In the series illustrated by the upper curve the strength of the conditioning stimulus (1·54 times threshold) was just maximal for Group 1. Since the facilitation appeared with an approximately half-maximal Group I volley and did not increase when the highest threshold Group I fibres were added to the conditioning volley, it is concluded that the facilitation was caused by Ib impulses (cf. Eccles *et al.* 1957*a*). The increment in facilitation at 3·04 and 6·1 times threshold may be attributed to

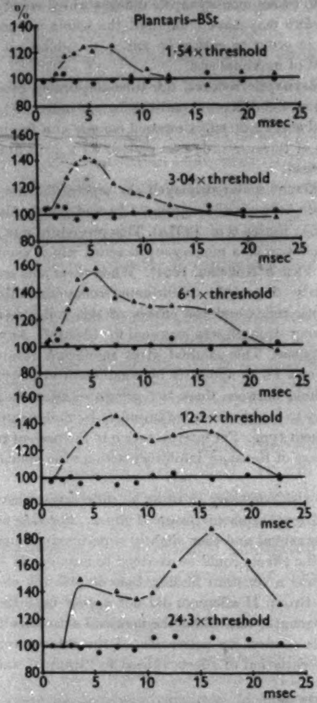

Fig. 1. Effects of conditioning volleys in the nerve to plantaris on the monosynaptic reflex from the nerve to posterior biceps-semitendinosus. In each instance 100% on the ordinates represents the unconditioned amplitude of the test monosynaptic reflex. Conditioned amplitude of monosynaptic test reflex, expressed as percentage of control amplitude, is plotted as a function of time interval between incoming conditioning and testing volleys which were recorded at the dorsal root entry zone. The experiment was made on a decerebrate cat and effects examined before (●) and after spinal transection (▲).

The curves were obtained at different strengths of conditioning stimuli. For each curve this strength is expressed in multiples of threshold strength.

impulses in Group II fibres (cf. Eccles & Lundberg, 1959). The late phase of facilitation that appeared with increase of stimulus strength from 12·2 to 24·3 times threshold was due to Group III impulses since a stimulus of 8–10 times threshold strength suffices to excite all Group II fibres (Eccles & Lundberg, 1959). Hence it can be concluded that the facilitatory actions by Ib, II and III impulses show a release after cord section. The synaptic actions by these afferents are mediated by interneurones and the only plausible interpretation of these findings is that, in the decerebrate preparation, these interneurones are tonically inhibited by descending impulses. In two control experiments it was found that a similar release occurred after an upper spinal (C1) section; hence these descending impulses must originate in the brain stem.

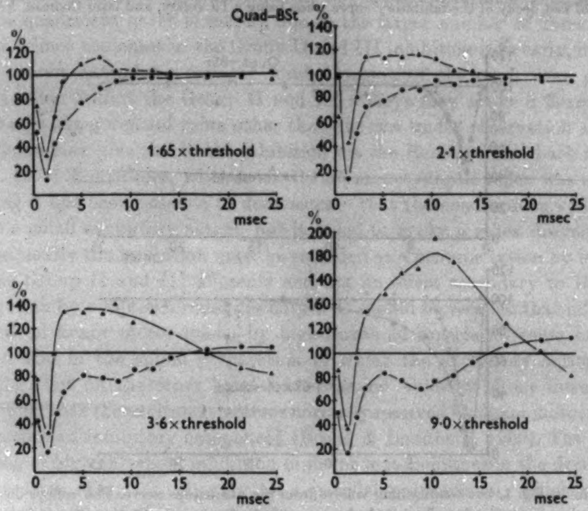

Fig. 2. As in Fig. 1, but conditioning volleys in the nerve to quadriceps. Conventions as for Fig. 1.

In the curves of Fig. 2 the monosynaptic reflex from posterior biceps-semitendinosus was preceded by a volley from the antagonist muscle, quadriceps. The quadriceps volley displayed complete separation into Ia and Ib components (cf. Bradley & Eccles, 1953; Eccles *et al.* 1957 *a*; Laporte & Bessou, 1957). The conditioning volley at 1·65 times threshold included approximately half, and, at 2·1 times threshold, the whole Ib component. From ten experiments it was noted that the magnitude of the Ia inhibition does not appreciably alter after spinal transection. In the upper curves of Fig. 2 the decay of inhibition is faster after cord section and is succeeded by a phase of facilitation. This is the characteristic Ib facilitatory action and its absence before transection of the cord corresponds with the findings in the upper curve of Fig. 1.

Lloyd (1946*a*) originally described a 'direct' inhibition curve that changed exponentially from its maximum with a time constant of about 4 msec. When Laporte & Lloyd (1952) later found an initial more rapid phase of decay from the maximum, they attributed this deviation to the superposition of an excitatory action by Ib impulses. However, Bradley & Eccles (1953) observed the initial fast decay of the inhibitory curve when using a Ia volley, and later Coombs, Eccles & Fatt (1955) concluded that the brief action was due to the increased subsynaptic currents through the IPSP elements, the later more prolonged action being attributable to the hyperpolarization of the whole post-synaptic membrane. In the present investigation a rapid non-exponential decay of the direct inhibitory curve was often observed. Figure 3 shows curves obtained on conditioning the monosynaptic reflex from BSt with volleys from the quadriceps nerve. This nerve displayed complete separation into Ia and Ib volleys and at a stimulus strength of 1·15 times threshold less than half the Ia fibres were activated, whereas at 1·69 times threshold stimulation was supramaximal for Ia and included about half the Ib afferents. The double composition of these inhibitiory curves is evident also before cord section, and this is of particular interest because, since Ib excitatory actions are absent in the decerebrate state, the composite curve cannot be caused by impulses in such Ib fibres as may 'contaminate' the Ia volley (cf. Laporte & Bessou, 1957; Eccles *et al.* 1957*a*).

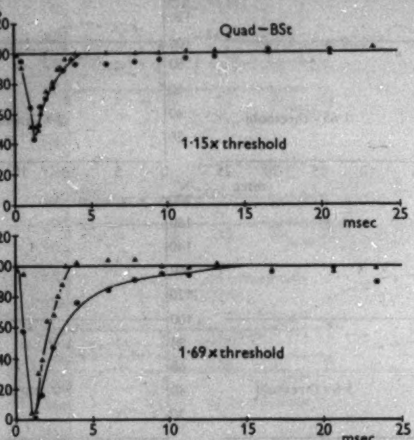

Fig. 3. As in Fig. 1, but conditioning volleys from the quadriceps nerve. The volleys displayed complete separation into Ia and Ib components. A stimulus of 1·15 times threshold strength (upper curve) evoked a Ia volley of less than half the maximal size. The conditioning volley evoked at 1·69 times threshold (lower curve) included about half of the Ib fibres. Conventions as for Fig. 1.

In the lower curves of Fig. 2 a later phase of inhibition appeared at Group II strength and reversed to facilitation after section of the spinal cord. When the stimulus strength was raised to 23·5 times threshold in order to include Group III fibres, this late phase of inhibition increased considerably (not illustrated). This late phase of inhibition was regularly found when the conditioning stimuli were applied to the quadriceps nerve. Though it is not observed in Fig. 1, it occurred occasionally when the nerves to plantaris, gastrocnemius, soleus and flexor digitorum longus were given conditioning stimuli at Group III strengths. Possibly its regular appearance on stimulation of the quadriceps nerve is merely due to the larger number of fibres in this nerve. Since the onset of the Group II and III inhibition is so early, its pathway cannot extend beyond the lumbar region of the cord. One possible explanation is that the Group II and III volleys may evoke a flexor reflex discharge along ventral roots other than the one under observation and this discharge may give rise to the inhibition via the Renshaw feed-back (Eccles, *et al.* 1954) Sometimes, when a very small monosynaptic reflex was used for testing it has been possible to demonstrate that the conditioning volley may have a small excitatory action, but it failed to evoke a reflex discharge and consequently the inhibition must be regarded as a genuine action by impulses in the Group II and III afferents and not an effect secondary to Renshaw activation by a motoneuronal discharge. It should be recalled that inhibitory action on flexor motor nuclei by high-threshold muscle afferents has been found also in the spinal preparation in which the excitatory actions were lacking; but on the other hand there was no evidence from intracellular recording that the excitatory actions normally received by flexor motoneurones concealed an inhibitory component (Eccles & Lundberg, 1959). The present finding of high threshold inhibition in flexor motoneurones in the decerebrate preparation (Fig. 2*C*, *D*) may be a parallel phenomenon, which becomes detectable when the excitatory pathway is depressed in the decrebrate animal. As regards the functional significance of this inhibition the possibility exists that the central nervous system has two pathways at its disposal by which impulses in high-threshold muscle afferents may act on flexor motoneurones. However, the fact that the inhibitory component is rarely evoked except on stimulation of the very large quadriceps nerve necessitates caution. Undoubtedly the single-volley technique has its limitations and experiments with adequate stimulation would be valuable.

The action by impulses in high-threshold muscle afferents on flexor motoneurones is further illustrated in Figs. 4 and 5. Six posterior biceps-semitendinosus motoneurones were impaled by a micro-electrode before cord section and another six afterwards. In none of the cells before transection was there any significant addition to the monosynaptic EPSP when the stimulus strength was raised above Group I strength (Fig. 4, *F–H*). On the other hand, after spinal transection (Fig. 5, *A–K*) the characteristic late excitatory action appears at Group II strength (D) and grows with inclusion of more

Group II fibres (*E–G*). A late EPSP is added by impulses in Group III fibres (*J*) leading to impulse generation (*K*). On stimulation of the quadriceps nerve there is no evidence of Ib excitatory action (Fig. 4, *J–K*), whereas a Ib EPSP can be clearly seen after section of the spinal cord (Fig. 5, *O–P*). A late inhibitory potential appears as a delayed decay of the Ia IPSP (Fig. 4, record *N*). It is uncertain whether the large increase of the late IPSP (Fig. 4, *P*) is contributed by the lowest threshold Group III afferents; but with further increase of stimulus strength (*Q–T*) an additional IPSP appears that must be

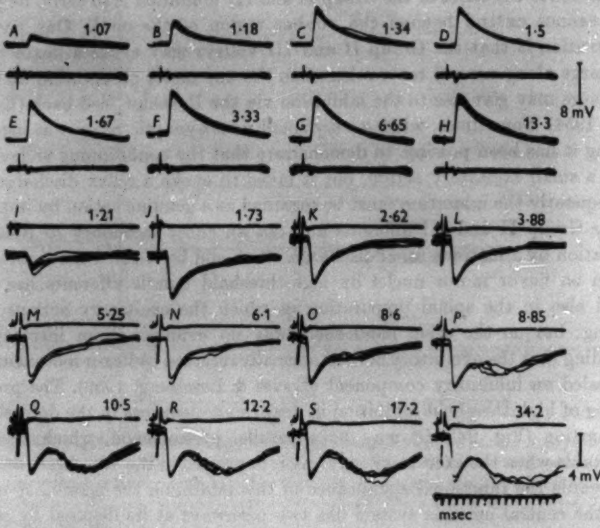

Fig. 4. Intracellular recording, with micro-electrode filled with 0·6 M-K_2SO_4, from a motoneurone of the posterior biceps-semitendinosus nerve. *A–H*, responses evoked by stimulation of the nerve to posterior biceps-semitendinosus; *I–T* by stimulation of the nerve to quadriceps. The stimulus strengths relative to threshold strength are indicated in the records. In the triphasic records of the dorsal root volley, negativity is unconventionally signalled by a downward deflexion. The animal was decerebrated.

due to Group III fibres. After spinal transection Group II and Group III contribute the usual late EPSP's (Fig. 5, *R–Z*) leading to generation of impulses in *Y* and *Z*.

The findings in Figs. 4 and 5 were consistent in all the cells investigated and correspond in detail with the results obtained in the experiments in which the monosynaptic reflex was used as an index of the motoneuronal excitability. The steadier base line in Fig. 4, compared with Fig. 5, suggests that the flexor motoneurones in the spinal animal receive a more intense background bombardment from interneurones than in the decerebrate state.

Action of impulses in joint and skin afferents. Figure 6 *A* and *B* shows the effect of conditioning volleys in the posterior nerve to the knee joint and in the superficial peroneal nerve, respectively. In the decerebrate preparation there was little or no facilitatory effect even when the joint nerve was stimulated at strengths 25 times threshold, but after transection of the cord a large facilitation, as in Fig. 6 *A*, could regularly be evoked (cf. Eccles & Lundberg, 1959). On the other hand, a conditioning volley from the superficial peroneal nerve usually gave some facilitation mixed with inhibition, as in Fig. 6 *B*, and after cord section there occurred a large increase of the facilitatory action.

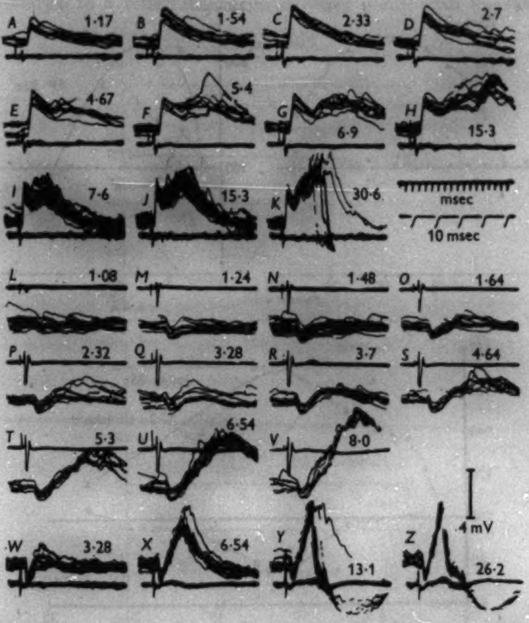

Fig. 5. Intracellular records, with micro-electrode filled with 10·6 M-K_2SO_4, from a motoneurone of the posterior biceps-semitendinosus group. The records were obtained in the same experiment as those of Fig. 5 but after transection of the spinal cord at L1.

A–K show responses evoked by stimulation of the posterior biceps-semitendinosus nerve, *L–Z* by stimulation of the quadriceps nerve. Records *I–K* and *W–Z* were obtained at the slower sweep speed.

In some animals, particularly those anaesthetized with ether, a small inhibitory effect was produced by impulses in the nerve from the knee joint, and after cord section it reversed to facilitation (Fig. 6*C*). This effect resembles that found when conditioning by Group II and III muscle afferents, and presumably they are related phenomena since they are always found in the same animals.

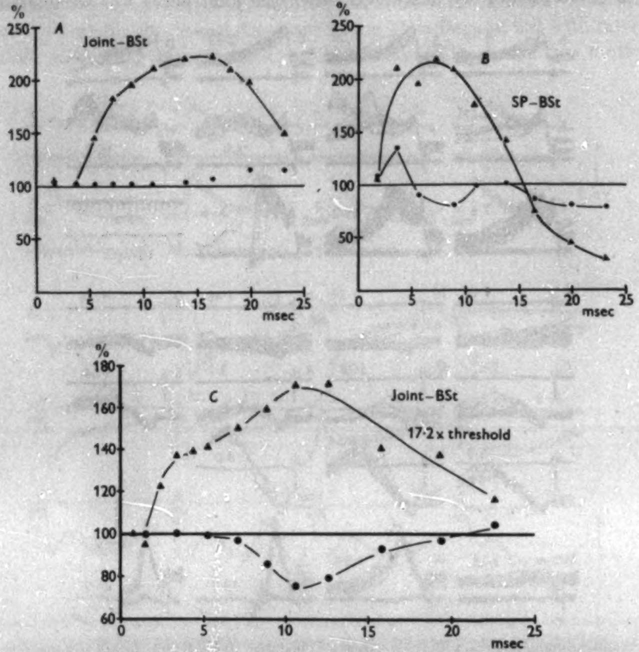

Fig. 6. As in Fig. 1, but conditioning volleys in the posterior nerve to the knee joint (*A* and *C*) and the superficial peroneal nerve (*B*). Curves *A* and *B* were obtained from the same animal. Conventions as for Fig. 1.

Extensor nuclei

Action of impulses in muscle afferents. In Fig. 7 are shown curves obtained when the monosynaptic reflex to the ankle extensor, gastrocnemius-soleus, was conditioned by volleys in the nerve to another extensor muscle, flexor digitorum longus (FDL). A maximal Group I volley (1·55 times threshold) produced a small inhibition of uncertain time course, which was probably a Renshaw effect caused by the monosynaptic reflex in the motor fibres to FDL, though the early phase might be Ib inhibition. The important finding in

Fig. 7 is that with increase of stimulus strength to include Group II (5·1 times threshold) and Group III (10·3–26 times threshold) there was no evidence that these impulses contributed inhibitory action in the decerebrate state. On the other hand, after spinal transection the usual inhibitory effect occurred at all strengths of stimulation. It has already been demonstrated that this inhibitory effect does occur in the spinal animal, even if the conditioning volley does not give rise to a reflex discharge in flexor nuclei with the consequent Renshaw inhibition (Eccles & Lundberg, 1959). Consequently, this experiment shows a release of inhibitory action evoked by impulses in Ib, II and III fibres.

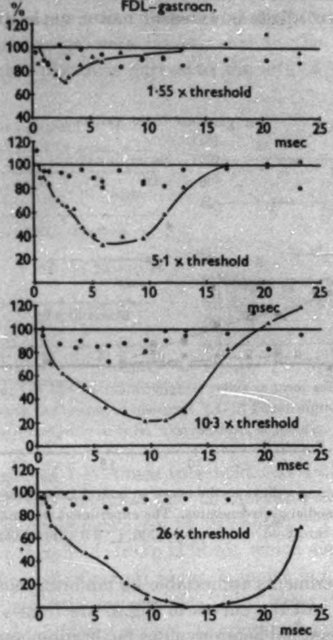

Fig. 7. As in Fig. 1, but the testing monosynaptic reflex from gastrocnemius-soleus, and the conditioning volleys in the nerve to flexor digitorum longus. Conventions as for Fig. 1.

There was a similar release of inhibitory action by Group II and III volleys on an extensor motor nucleus when the conditioning volley was in the nerve to an extensor muscle, operating at another joint (quadriceps–gastrocnemius) or in the nerve to a flexor muscle (BST–gastrocnemius). However, only in a few cases were inhibitory effects completely absent in the decerebrate preparation. If the animals had been anaesthetized with pentothal until the decerebration, there was regularly inhibitory action by Group III volleys, and sometimes a little by Group II volleys, in all the nerves investigated. If ether had been used, then the inhibitory effects in decerebrate cats were always less and sometimes absent. In a later series of experiments made on cats decerebrated under ether, it was regularly found that maximal Group III volleys in the nerves to gastrocnemius-soleus, FDL or plantaris did not in the non-spinal state evoke inhibitory effects in extensor motor nuclei (Holmqvist & Lundberg, unpublished).

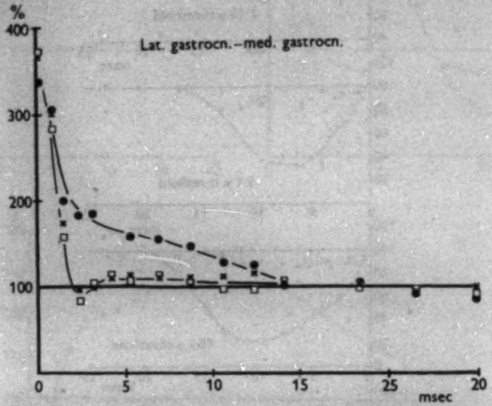

Fig. 8. Effects of conditioning volleys in the nerve to lateral gastrocnemius-soleus on the monosynaptic reflex from medial gastrocnemius. The experiment was made on a non-spinal decerebrate cat. ● 1·33 × threshold, × 1·8 × threshold, ☐ 9·0 × threshold.

In some of the experiments appreciable Ib inhibitory actions were found in the non-spinal state. All the curves in Fig. 8 are from a non-spinal decerebrate preparation. Maximal heteronymous facilitation, and hence stimulation of all Ia fibres, was obtained with a conditioning volley at 1·33 times threshold. A decay of facilitatory action faster than the expected exponential curve (Lloyd, 1946*a*) may indicate that some Ib fibres were stimulated at this strength. At 1·8 times threshold (maximal for Group I) the Ib inhibitory action has increased considerably, whereas there was no further change of the curve with the addition of the Group II volley to the conditioning volley (9 times threshold). Appreciable Ib inhibitory actions were sometimes found in animals in which Group III volleys had no inhibitory action. On the other hand, Ib effects could be suppressed in the non-spinal animal when Group III volleys had some inhibitory action. Hence, it seems reasonable to assume that the interneurones mediating these two groups of actions are independently controlled.

Of considerable interest is the finding that there are different patterns of Ib inhibitory action in the decerebrate and the spinal preparations. The spinal animal is characterized by very large actions from flexor digitorum longus (Eccles *et al.* 1957*c*; Eccles & Lundberg, 1959), whereas in the decerebrate preparation the Ib inhibitory actions from FDL are the smallest. The curves in Fig. 9 illustrate an experiment in which Ib actions from the nerves to plantaris, FDL and quadriceps were investigated in the gastrocnemius-soleus nucleus. Of the three plantaris curves to the left, *A* was obtained with a conditioning strength of 1·17 times threshold. At 1·65 times threshold (*B*) the Group I volley was maximal and the increase of inhibitory action in comparison with the upper curve can be attributed to Ib impulses. That they were not due to low-threshold Group II fibres, which may overlap in threshold with the highest-threshold Group I fibres, is shown in *C*, where an increase of the strength of the conditioning stimulus to 2·7 times threshold did not bring about any increased inhibitory action. It appears from the curves that there was only a moderate increase in inhibition after section of the cord. On the other hand, with a conditioning FDL volley in Fig. 9*D*, the inhibition before cord transection was smaller than with plantaris, but it was increased much more by the transection, and similar results were obtained with a conditioning quadriceps volley (Fig. 9*F*).

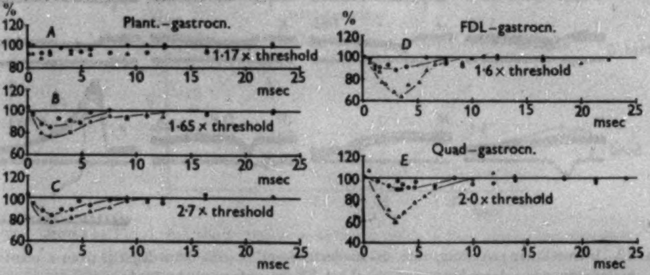

Fig. 9. As in Fig. 1, but the testing monosynaptic reflex is from gastrocnemius-soleus, and the conditioning volleys in the nerves to plantaris, (*A–C*) flexor digitorum longus (*D*), and quadriceps (*E*). Further description in text. Conventions as for Fig. 1.

This is further illustrated in Figs. 10 and 11, showing intracellular records from two plantaris motoneurones in the same animal. A very weak homonymous volley in plantaris caused the EPSP in Fig. 10*P*. With increased strength of stimulation an impulse was regularly generated in this motoneurone, hence it was not possible to analyse Ib actions from the plantaris nerve. Records *A–D* show the effects of volleys evoked in the nerve FDL by different stimulus strengths. The heteronymous monosynaptic EPSP (cf. Eccles *et al.* 1957*b*) had its usual characteristic time course in *A* and did not change when high-threshold Group I fibres were included (*B—D*). This is in striking contrast to what is found in the spinal cat, where increase of the Group I volley causes

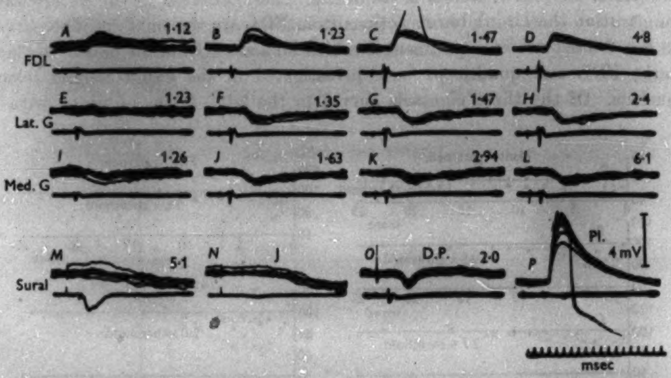

Fig. 10. Intracellular recording, with micro-electrode filled with 0·6 M-K_2SO_4, from a plantaris motoneurone in a decerebrate non-spinal cat. The records were obtained on stimulation of the nerves to following muscles: *A–D*, flexor digitorum longus (FDL); *E–H*, lateral gastrocnemius-soleus (Lat. G); *I–L*, medial gastrocnemius (med. G); *O*, extensor digitorum longus and tibialis anticus (D.P.); and *P*, plantaris. To evoke the response in *M* the sural nerve was stimulated and in *N* the posterior nerve to the knee joint.

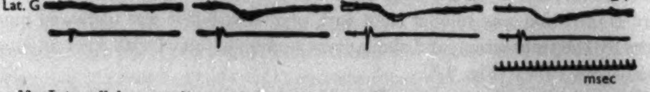

Fig. 11. Intracellular recording, with micro-electrode filled with 0·6 M-K_2SO_4, from a plantaris motoneurone. The records were obtained in the same experiment as those of Fig. 10, but after transection of the spinal cord at L1. The nerves to the following muscles were stimulated: *A–D*, plantaris (Pl); *E–H*, flexor digitorum longus (FDL); and *I–L*. lateral gastrocnemius-soleus (Lat. G).

a Ib IPSP to be superimposed on the monosynaptic EPSP, as in Fig. 11, *F–G* (cf. Eccles *et al.* 1957*c*). On the other hand, in Fig. 10 Ib IPSPs were evoked before the cord section from the nerves to lateral gastrocnemius-soleus (*E–H*) and to medial gastrocnemius (*I–L*), and they were of the same order of magnitude as those evoked by the lateral gastrocnemius-soleus volley in another motoneurone after the cord had been divided (Fig. 11, *I–L*). There was also a relatively large homonymous Ib IPSP from plantaris (Fig. 11, *B–D*). Hence both the experiments with intracellular recording and those with conditioning of monosynaptic reflexes have indicated that the Ib inhibitory actions from FDL are more effectively suppressed in the decerebrate preparation than those from the other ankle extensors.

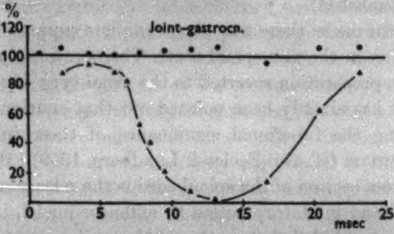

Fig. 12. As in Fig. 1, but the testing monosynaptic reflex is from gastrocnemius-soleus, and the conditioning volleys in posterior nerve to knee-joint.

Action by impulses in joint and skin afferents. The effects of conditioning volleys in joint and skin nerve afferents are also different before and after spinal section. In Fig. 12 a conditioning volley in the posterior nerve to the knee joint had no effect before section of the spinal cord but afterwards the same volley gave pronounced inhibition. Renshaw inhibition from the discharge in flexor nuclei may have contributed to this action but is not the exclusive cause of it, because strong inhibitory effects were found also in those cases in which no reflex discharge was noted in the ventral roots on stimulation of the joint nerve. For investigation of effects from cutaneous afferents we stimulated the sural or the superficial peroneal nerve. There was usually, but not always, some inhibition action also in the non-spinal state, but the effect always increased very considerably after spinal transection. In the non-spinal state there was always a close parallelism in the degree of suppression of synaptic actions from high-threshold muscle afferents, skin and joint afferents.

DISCUSSION

In this investigation Job's (1953) finding has been confirmed, that in decerebrate and spinal preparations there are very considerable differences in synaptic actions evoked in motoneurones by impulses in somatic afferents.

Job used 'supramaximal' conditioning volleys in muscle nerves and found, for extensor nuclei, a reversal from excitation to inhibition after cord section. In the present series of experiments there was never any excitatory action in extensor motor nuclei on conditioning with volleys in Group II and III muscle afferents. However, after interruption of the pathways from higher centres, there was always a release of inhibitory action. Though Job's conditioning volley may have included C fibres, the onset of excitatory action was too early to be explained in this way. It must not necessarily be assumed that the excitatory action (Job, 1953) was caused by impulses in high-threshold afferents. The possibility that Ia afferents under some conditions may have polysynaptic excitatory actions cannot be entirely excluded (Eccles, Eccles & Lundberg, unpublished).

In flexor motor nuclei there was often complete suppression of polysynaptic excitatory action in the non-spinal state. The occasional slight inhibition in the decerebrate preparation reverted to the usual type of excitation on spinal transection. It has already been pointed out that caution must be observed when evaluating the functional significance of these inhibitory effects in flexor motoneurones (cf. also Eccles & Lundberg, 1959). However, the dominating result from section of the spinal cord is the release of excitatory action to flexor nuclei and inhibitory action to extensor nuclei. In agreement with Job (1953), it is concluded that section of the cord removes a tonic inhibitory action which suprasegmental centres exert on interneurones mediating actions from muscle afferents.

What functional significance is to be attributed to the existence of two systems of muscle-spindle afferents with different patterns of synaptic actions on motoneurones? How can the detailed patterns of Ia connexions assert themselves among the large non-specific actions of Group II afferents? It is important to know the nature of the influence of the higher centres on the transmission of impulses across the Group Ia and the Group II reflex arcs.

It has now been demonstrated that the interneurones mediating Group II actions are very effectively inhibited from suprasegmental levels. Hence, it is possible that, under circumstances in which the Ia system operates optimally in reflex control of movements, the impulses in Group II afferents are prevented from exerting any action on the motoneurones because of the inhibitory bias on their interneurones. There is no evidence that the Ia pathways are under a similar control. The Ia excitatory actions are exerted monosynaptically and there is no evidence of any change after spinal transection. On the other hand, it is known that there is an interneurone in the inhibitory pathway (Eccles, Fatt & Landgren, 1956; Eccles & Lundberg, 1958*b*), but spinal transection has no appreciable effect on Ia inhibition (Figs. 2, 3 and Job, 1953).

If, as suggested above, Group II impulses are prevented from exerting any action during locomotion, it remains to discover their functional significance. It was noted that there was a close parallelism between the effect of spinal transection on synaptic actions exerted by Group II muscle afferents and those of Group III muscle afferents as well as skin and joint afferents. This finding supports the hypothesis (Eccles & Lundberg, 1959) that the flexor reflex action exerted by Group II afferents is part of the general flexion reflex, which presumably is of protective importance and functions mainly when the animal is at rest. In the alerted animal, on the other hand, it would be beneficial if these reflexes were prevented, since they would limit the animal's capacity for expedient action.

Of considerable interest is the finding that the interneurones mediating Group Ib actions, both excitatory and inhibitory, are also tonically inhibited in the decerebrate preparation. At least, with respect to inhibition, it was possible experimentally to differentiate this control from that exerted on the interneurones mediating the actions of the general flexion reflex. It is not difficult to suggest a physiological significance for this control. It has been proposed that the Ib system may be of importance in terminating the extension and initiating the flexion phase of stepping (Eccles & Lundberg, 1959). It has been seen (Figs. 9–11) that the suprasegmental control is particularly effective with those interneurones relaying the Ib impulses from flexor digitorum longus. If Ib impulses from flexor digitorum longus should be of special importance in the reflex regulation of stepping, a control from higher centres of these reflex actions would be needed in order to regulate the movements of stepping and running. A control of Ib interneurones would presumably also be of importance in voluntary movements of various strengths.

The central nervous system apparently has powerful possibilities to regulate the central action of individual receptor systems. The control of the Ia system is via the γ-efferents which change the sensitivity of the receptor itself (Leksell, 1945; Hunt & Kuffler, 1951; Granit & Kaada, 1952; Eldred, Granit & Merton, 1953). A more complex situation prevails with the actions of Group II fibres with flower-spray endings or muscle spindles, since in this case both the receptor and the interneurones are controlled. There is no knowledge of any control of the receptor with other systems, and regulation may be entirely at the interneuronal level.

With regard to the control of interneurones of the flexion reflex, it should be recalled that there are several ascending pathways which are influenced by the same system of afferents. Two pathways have been found which are polysynaptically excited by impulses in Group II and III muscle afferents, by skin and by joint afferents from very wide receptive fields (Laporte, Lundberg & Oscarsson, 1956; Holmqvist, Lundberg & Oscarsson, 1956; Oscarsson, 1958). Neurones of another pathway, the ventral spinocerebellar tract, receive powerful inhibitory actions from these afferents as well as monosynaptic excitatory actions from Ib afferents (Oscarsson, 1957). Since all these pathways are influenced by the same system of afferents that give rise to the flexion reflex, there is a possibility that they are informative with respect to this reflex; they may, for example, be afferent links in the suprasegmental regulation of the inhibitory bias on the interneurones mediating the flexion reflex (cf. Holmqvist, Lundberg & Oscarsson, 1959).

SUMMARY

1. The synaptic actions by impulses in muscle, joint and cutaneous afferents have been compared in decerebrate cats before and after section of the spinal cord.

2. In flexor motor nuclei of the non-spinal animal, impulses in Group II and III muscle and joint afferents have little or no excitatory action, whereas large actions appear after spinal transection. Excitatory actions by impulses in cutaneous afferents also increase considerably after cord section. Correspondingly, in extensor motor nuclei inhibitory action by the same systems of afferents appears or, if already present, increases after spinal transection. It is concluded that the interneurones mediating the actions of the general flexion reflex are tonically inhibited from the brain stem in the decerebrate preparation.

3. A similar release on spinal transection is found for the excitatory and inhibitory actions evoked by Ib impulses, showing that interneurones of the Ib pathways are also tonically controlled from the brain stem. The interneurones mediating Ib inhibitory actions from flexor digitorum longus are more effectively controlled from higher centres than those mediating Ib effects from other ankle extensors.

4. Some findings suggest that the suprasegmental control of the interneurones mediating Ib inhibitory actions is functionally independent of that controlling interneurones mediating the actions of the general flexion reflex.

5. There has been no evidence of any suprasegmental control of interneurones mediating Ia inhibitory actions.

6. The results are discussed mainly in relation to the significance of the different receptor systems from muscle in the reflex taxis of the animal.

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INHIBITION OF ACID GASTRIC SECRETION IN THE RAT BY CHORIONIC GONADOTROPHIN

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It has been found repeatedly that preparations of chorionic gonadotrophin inhibit gastric secretion (Culmer, Atkinson & Ivy, 1939; Friedman & Sandweiss, 1941; Code, 1951) and Huff, Risley & Barnes (1950) showed that even highly purified gonadotrophins possessed this action. The question thus arises whether antisecretory activity resides in the gonadotrophin molecule itself. Huff *et al.* considered this to be unlikely, since they found that gonadotrophin preparations of different stages of purity and varying fourfold in gonadotrophic activity produced about the same degree of secretory inhibition. In this paper the problem of the antisecretory action of chorionic gonadotrophin has been reinvestigated by a new quantitative assay method for inhibitors of gastric secretion (Ghosh, 1958) based on the continuous perfusion of the stomach of the rat. Two purified gonadotrophin preparations were thus tested and their gonadotrophic and antisecretory effects correlated.

METHODS

Rats of 150–400 g were anaesthetized with 25% urethane 0·5–0·7 ml./100 g intramuscularly. Drugs were injected intravenously through a cannula in the jugular vein. In some experiments the blood pressure was recorded by means of a mercury manometer from the carotid artery.

For the assay of gonadotrophic activity the method of Loraine (1950) based on the prostatic weight of rats was used with slight modifications.

Acid gastric secretion was measured by a method which is described in detail elsewhere (Ghosh & Schild, 1958). Briefly, the stomach of a rat anaesthetized with urethane is perfused with diluted sodium hydroxide by way of the oesophagus, and the pH of the fluid emerging from a cannula in the pylorus is registered graphically. During its passage through the stomach the perfusate collects sufficient buffer to act as an approximately linear buffer system, which changes its pH according to the amount of acid secreted.

The following preparations of chorionic gonadotrophin were used: (1) a commercial preparation ('Pregnyl'; Organon, Ltd.); (2) a purified preparation supplied by Paines and Byrne Ltd., which had a stated potency of 1200 i.u./mg—this preparation was dissolved in distilled water, distributed in ampoules to contain 5 mg each and freeze-dried (preparation *A*); (3) a purified preparation supplied by Organon Ltd. containing 3000 i.u./mg—this preparation was put up in ampoules under nitrogen in 2 mg lots (preparation *B*). Histamine acid phosphate was used, and its activity is expressed in terms of the base.

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RESULTS

Effect of chorionic gonadotrophin on acid secretion

Intravenous injections of chorionic gonadotrophin inhibit the stimulant effect of histamine on acid secretion. In the experiment illustrated in Fig. 1 intravenous doses of 150 and 300μg histamine produced transient stimulation of acid secretion; and intravenous injection of 600 i.u. chorionic gonadotrophin (Pregnyl) produced no obvious effect of its own but reduced the response to 300μg histamine administered 10 min later. The response to a further dose of histamine was also reduced.

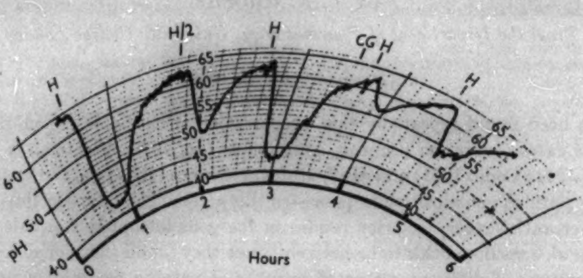

Fig. 1. Continuous pH record of acid gastric secretion in the rat. Original circular chart. Effect of an intravenous dose of 600 i.u. chorionic gonadotrophin (*CG* Pregnyl) on histamine-induced gastric secretion. *H* = histamine 300 μg, *H*/2 = 150 μg.

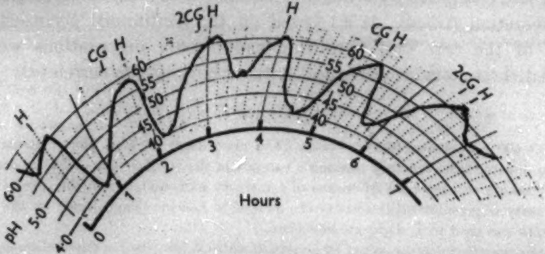

Fig. 2. Acid gastric secretion as in Fig. 1. Effects of intravenous doses of 1500 (*CG*) and 3000 (2 *CG*) i.u. chorionic gonadotrophin (Pregnyl) on acid secretion by histamine (*H* = 300 μg) given 10 min later. Note reversibility of inhibition.

The inhibitory effect of chorionic gonadotrophin is graded according to dose as shown in Fig. 2. In this experiment, on a less sensitive preparation, 1500 i.u. Pregnyl produced a slight, and 3000 i.u. a conspicuous inhibition, both effects being reversible. Full reversibility of the inhibitory effects was however the exception rather than the rule and in most instances a single dose of chorionic gonadotrophin produced a long-lasting inhibition affecting several doses of histamine (Fig. 3). For this reason it was decided to administer to each rat only a single dose of chorionic gonadotrophin for quantitative assays. The usual procedure was to give two successive doses of histamine, usually 100–300μg, according to sensitivity, followed by an intravenous injection of chorionic gonadotrophin, and 10 min later by a further injection of the same dose of histamine. The difference in the pH deflexions of the histamine curve before and after gonadotrophin was taken as index of secretory inhibition.

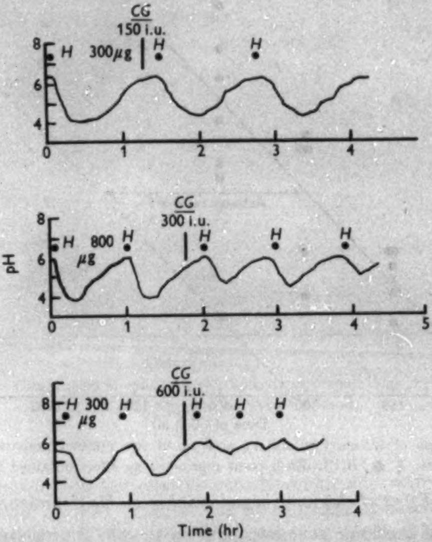

Fig. 3. Continuous pH record of acid gastric secretion in the rat. Record transposed to linear coordinates. Graded inhibition of histamine (*H*) secretion produced by varying doses of chorionic gonadotrophin (*CG*, preparation *A*). In each of the three experiments on different rats the same dose of histamine was used throughout. Note persistence of inhibition.

Comparison of gonadotrophic and antisecretory activity of gonadotrophins

Preliminary experiments having indicated that the gonadotrophin preparations *A* and *B* differed considerably in secretory inhibitory activity, they were selected for a quantitative assay in which their gastric and gonadotrophic effects were compared. For the secretory assay both preparations were used at three levels of dosage in the ratio 1:2:4. Figure 4 shows that the log. dose-response curves of the two preparations were parallel and approximately linear. Preparation *A* showed considerably greater secretory-inhibitory activity in relation to its stated gonadotrophic activity than preparation *B*, the ratio being 5·5. In order to check the manufacturers' assays a direct comparison of the gonadotrophic activity of two preparations was carried out by the rat prostate method. This comparison, which is shown in Fig. 5, gave an activity ratio of 1·27, which was not significantly different from 1. It thus seems clear that the gonadotrophic and gastric inhibitory activities of the two preparations do not coincide.

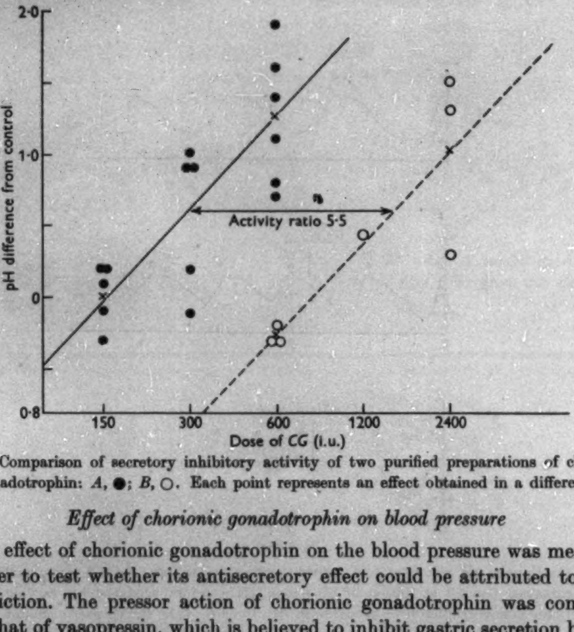

Fig. 4. Comparison of secretory inhibitory activity of two purified preparations of chorionic gonadotrophin: *A*, ●; *B*, ○. Each point represents an effect obtained in a different rat.

Effect of chorionic gonadotrophin on blood pressure

The effect of chorionic gonadotrophin on the blood pressure was measured in order to test whether its antisecretory effect could be attributed to vasoconstriction. The pressor action of chorionic gonadotrophin was compared with that of vasopressin, which is believed to inhibit gastric secretion by way of vasoconstriction (Cutting, Dodds, Noble & Williams, 1937; Ghosh, 1958). It was found that chorionic gonadotrophins produced a negligible rise of blood pressure in the urethanized rat, 6000 i.u. of gonadotrophin (preparation *A*) producing an effect equal to 0·5 m-u. vasopressin. Since this dose of gonadotrophin contains approximately 10 maximal gastric inhibitory doses whilst 0·5 m-u. vasopressin contains only about 1/100th of an effective gastric inhibitory dose, the discrepancy between the pressor and antisecretory effects of the two substances is at least a thousandfold. It thus seems unlikely that the gonadotrophin inhibits gastric secretion by way of vasoconstriction.

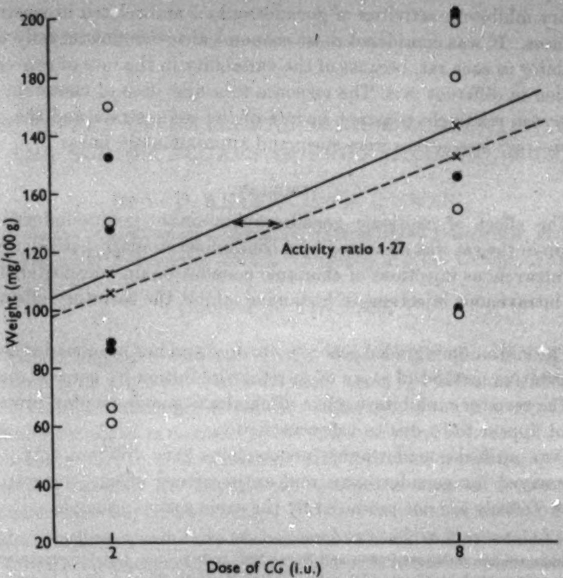

Fig. 5. Comparison of gonadotrophic activity of preparations *A* (●) and *B* (○) by the prostate weight method.

DISCUSSION

It has been shown that the relative gonadotrophic and secretory inhibitory activities of two purified preparations of chorionic gonadotrophin do not correspond. Our results thus support the conclusion of Huff *et al.* (1950) that the two activities are due to different though possibly closely related active principles. The evidence for two active principles is, however, not entirely conclusive, since the assay could possibly have been vitiated by the presence of an inhibitor in one of the samples.

Assuming two active principles, the simplest hypothesis is that the gastric effect is due to a contamination of the gonadotrophin preparations by urogastrone, the gastric secretory inhibitor present in normal urine (Gray, Wieczorowski, Wells & Harris, 1942). In favour of this view is the finding (M. N. Ghosh, R. A. Gregory & H. O. Schild, unpublished observations) that purified samples of urogastrone prepared by Gregory's (1955) method from normal male urine produced an inhibition of histamine-induced secretion in the rat of much the same character as that produced by gonadotrophins.

The present method seems suitable for the quantitative assay of the secretory-inhibitory activities of gonadotrophins and related urogastrone-like substances. It was considered most economical to administer only one dose of inhibitor to each rat, because of the variability in the rate of recovery from inhibition in different rats. The response to a first dose of chorionic gonadotrophin was relatively constant in rats of the same strain and the resulting log. dose–response curves were steep and approximately linear.

SUMMARY

1. The effect of chorionic gonadotrophin on histamine-induced gastric secretion in the rat was measured by a continuous stomach-perfusion method.

2. Intravenous injections of chorionic gonadotrophin administered 10 min before intravenous injections of histamine inhibit the secretory effects of the latter.

3. The inhibition is graded according to dose and has been made the basis of a quantitative method of assay of secretory inhibition by gonadotrophin.

4. The secretory inhibitory effect of chorionic gonadotrophin preparations does not appear to be due to vasoconstriction.

5. Two purified gonadotrophin preparations gave different activity ratios when assayed for gonadotrophic and antisecretory effects, suggesting that these two effects are not produced by the same active principle.

I am indebted to Dr H. O. Schild for his interest and advice throughout the present investigation. Thanks are due to Messrs Paines and Byrne Ltd. and Organon Ltd. for supplying the salt-free preparations of chorionic gonadotrophin, and to Mr T. D. Whittet, Chief Pharmacist, University College Hospital, for freeze-drying the ampoules. The work was aided by a grant from the Medical Research Council.

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THE EFFECT OF EXTERNAL SODIUM CONCENTRATION ON THE SODIUM FLUXES IN FROG SKELETAL MUSCLE

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Ussing (1949) suggested that part of the observed Na efflux in frog muscle might result from a process that he termed 'exchange diffusion', involving a sodium for sodium exchange across the membrane. Thus, if a carrier with a high affinity for Na were confined to the membrane and able to diffuse across it only when it had formed a complex with Na, there would be a continual movement of labelled Na through the membrane without necessarily any simultaneous consumption of energy. If such a mechanism existed, complete removal of Na from the outside of the membrane might be expected to eliminate a substantial fraction of the total efflux of labelled Na, unless the cation substituted for Na^+ could also form a complex with the carrier and thus move across the membrane. We therefore investigated the effect on the Na efflux of substituting choline or lithium for the sodium in the external medium, and found that in both cases the efflux was promptly reduced by about half when the sodium in the interfibre fluid was replaced by the foreign cation. This observation clearly warranted further analysis, and the changes in Na efflux were examined for test muscles with altered internal Na concentrations, and at various temperatures. The effect of partial replacement of external Na was also investigated and the resulting changes in Na efflux were compared with the parallel alterations in Na influx. The effect of removing external Na was found to add to that of removing external K, and the relative magnitude of the two effects was studied as a function of the Na content of the muscles. A preliminary report on this work was made at the XX International Physiological Congress (Swan & Keynes, 1956).

METHODS

Measurements of sodium fluxes

For measurement of effluxes, frog muscles were dissected free and exposed to Ringer's solution containing ^{24}Na for about 3·5 hr. This period was calculated as sufficient for the specific activity of

Na within the fibres to rise very close to that of the medium. The muscle was then transferred at 5 or 10 min intervals through a succession of test-tubes containing inactive Ringer's solution. The radioactivity leaving the muscle during each interval was measured. The amount of radioactivity leaving the muscle per unit of time, corrected for decay of the isotope, was plotted logarithmically against time. From displacements of the curve caused by changes in the ionic composition of the medium, changes in the rate of loss of sodium from the muscle fibres were calculated.

For determination of Na influx, the muscle was exposed to Ringer's solution containing ^{24}Na for 10 min, and was then passed through a series of samples of inactive Ringer's solution as before; finally it was ashed in nitric acid and its residual radioactivity was measured. By adding the residual count to the counts for successive samples in reverse order, plotting total count against time, and extrapolating to zero time, an estimate was obtained for the amount of radioactivity in the fibres at the moment of removal from the ^{24}Na solution. The time of exposure to ^{24}Na and the specific activity of the solution being known, this gave a value for the sodium influx.

Preparation of muscles

Sartorius muscles weighing 20–55 mg from *Rana temporaria* were used for most of the experiments. In a few cases (see below) other muscles of the lower extremity of *R. temporaria*, and sartorii of *R. pipiens* and of the toad *Bufo vulgaris*, were used. They were fixed with strands of cotton thread to racks of Pyrex glass rod at 1·0–1·1 × their length measured with the limb extended. Only muscles not visibly damaged during the dissection were used. All muscles were electrically excitable at the end of the experiment and nearly all were still free from any microscopically visible damage to the fibres. Occasionally one or two fibres showed areas of coagulation. When at the end of an experiment damage to more than two fibres was evident, the results of the experiment were rejected. At the end of each experiment, after inspection and testing, the muscle was trimmed of tendon, blotted with filter paper, weighed to 0·1 mg on a torsion balance, and transferred to a small volume of 0·1 N-HNO_3 for assay of residual activity.

Isolated sartorius and extensor longus dig. IV muscles of *R. temporaria*, slightly stretched with small weights, were suspended in various test solutions in optical cuvettes, and changes in length of the muscles were measured microscopically with a micrometer eyepiece. No significant changes in length were observed in any of the various media or sequences of media used in the course of this work. However, muscles fibrillate vigorously for 30–45 sec on exposure to a solution in which choline is substituted for sodium, and for a similar period on transfer back to ordinary Ringer's solution. This response is abolished by the addition of $2\cdot5 \times 10^{-5}$ M tubocurarine to the medium.

The experiments were performed in all months except August and September, on animals kept in captivity at 8–15° C usually for not more than 3 weeks.

Apparatus

The glass rack and attached muscle were placed in a test-tube containing 2 ml. of radioactive Ringer's solution, and subsequently in a series of test-tubes each containing 7 ml. of inactive Ringer's solution. Stirring was effected by moving the muscle 10–15 mm up and down along its long axis at a rate of one oscillation per second, by suspending the glass rack from an electromagnetically assisted pendulum (Palmer, 1900) or from a rotating cam driven by a small electric motor. The transference of the muscle from one solution to another simply involved lowering and replacing the test tube; this change required only 2 sec, so that the error in timing successive collection periods of 5–10 min was negligible.

Unless otherwise stated the experiments were performed at 16·5–20° C, and during the course of any one experiment the temperature did not change by more than 1° C. In experiments on temperature effects the solutions were equilibrated in a stirred, insulated water-bath whose temperature could be held constant to within 0·1° C over a range 0–20° C.

Solutions

Radioactive Ringer's solution was prepared from neutron-irradiated $NaHCO_3$, dissolved in a slight excess of 2 N-HCl and dried over pellets of NaOH. The resulting crystals of radioactive NaCl were weighed and incorporated into Ringer's solution of the composition shown in Table 1 (Solution B). The highest specific activity used was roughly 4 mc/m-mole, and in these experiments lasting several hours it was practicable to use each ^{24}Na sample over a period of 2·5 days.

Inactive Ringer's solution *A* (Table 1) was used for the initial period of washing out radioactivity in most of the experiments. Sodium-free Ringer's solution was prepared by substituting lithium or choline chloride for NaCl in equimolar amounts. In experiments in which the effect upon Na efflux of low-Na K-free media was investigated, solution *B* (Table 1) was used, the low-Na K-free version being prepared by substituting lithium or choline chloride for NaCl and omitting KCl. In some experiments (see below) the K concentration was increased to 10 mM leaving other components the same. Solutions more than 4 days old were seldom used, and choline Ringer's solution was always freshly prepared before use.

TABLE 1. Composition of solutions (mM)

Solution *A*		Solution *B*	
NaCl	111·2	NaCl	111·2
K_2HPO_4	1·14	Na_2HPO_4	1·14
KH_2PO_4	0·23	NaH_2PO_4	0·23
$CaCl_2$	1·8	KCl	2·5
		$CaCl_2$	1·8

Analytical

Radioactivity lost from the muscle over a measured interval of time was measured by transferring the 7 ml. of rinsing solution directly or after appropriate dilution into a liquid Geiger counter tube (20th Century, Type M6M). Counting rates did not exceed 1100 counts/min, so that although correction was always made for radioactive decay (the half-life of ^{24}Na being taken as 15·0 hr) it was not necessary to correct for coincidence loss. In the figures shown in this paper the diameter of the circles surrounding all those plotted points on which extrapolations or calculations of changes in Na efflux are based is equal to or greater than ± the S.D. of the counts; and, except for the latter part of each experiment, in most instances is greater than ± twice the S.D. Samples obtained during the transition periods following changes in medium, when Na efflux was changing rapidly, were counted with less accuracy. The background count averaged 8 counts/min.

Muscles were sometimes analysed for Na, K and water content by drying to constant weight at 105° C, ashing in platinum crucibles for 8–12 hr at 570° C, dissolving the ash with a very small drop of concentrated HCl, and diluting appropriately for flame photometry. The resulting solutions were compared with 100 μM-KCl and 50 μM-NaCl, by means of an EEL flame photometer. Pyrex glassware was used throughout.

RESULTS

The effect of Na-free solutions on the frog's sartorius

When sodium in the external medium is replaced by either of the monovalent cations lithium or choline (Fig. 1), the efflux of Na decreases rapidly over the next 15–20 min and thereafter more slowly, apparently as a single exponential function of time. On replacing the sodium, the efflux promptly returns towards the original rate. Substituting nitrate for chloride in the external medium does not alter Na efflux (cf. Edwards, Harris & Nishie, 1957).

This prompt and reversible reduction in Na efflux on transferring the muscle to a Na-free medium is reproducible over a period of several hours in a small sartorius muscle. Figure 2 shows the results of an experiment designed to test the reproducibility of the effect and the reliability of the method used in calculating the magnitude of the reduction in Na efflux. In this and subsequent figures lines have been fitted visually to the last three points obtained in Na Ringer's solution and to the points obtained after 15–20 min exposure to the experimental medium containing Li. The effluxes have then been

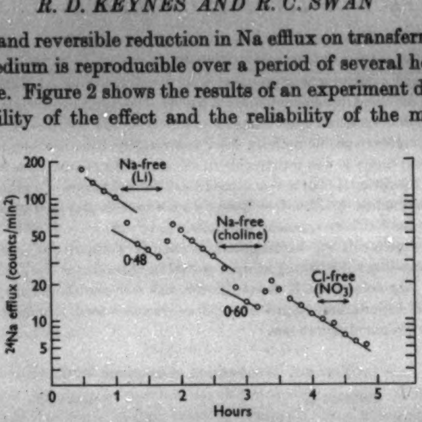

Fig. 1. The effect on the efflux of ^{24}Na from a small sartorius muscle weighing 30·2 mg of removing external Na and Cl. Temperature 17·5° C; Semi-log. scale. In this and subsequent figures the numbers under the counts indicate the fractions of the control efflux to which Na efflux has been reduced. For all counts used for an extrapolation, both here and in other figures, the diameter of the circle was greater than ±1 × S.D.

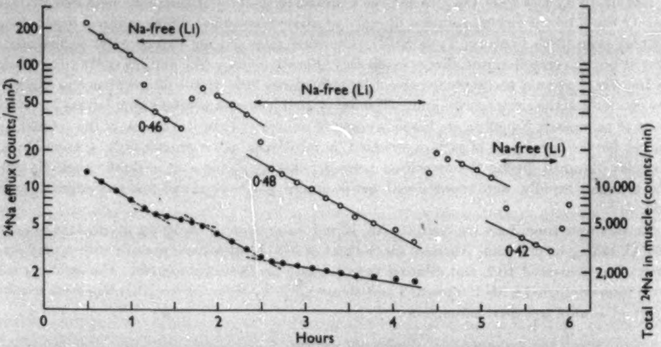

Fig. 2. The efflux of ^{24}Na from a sartorius muscle weighing 30·3 mg, exposed three times to Li Ringer's solution. Temperature 18·8° C. ○, efflux (counts/min^2, left-hand scale); ●, total radioactivity (counts/min, right-hand scale), calculated from the upper curve and from a count of the whole muscle at the end of the experiment. Semi-log. scale.

extrapolated to positions midway in time between the two sets of data, and their values at these positions expressed as ratios. These ratios represent estimates of the fraction of the original Na efflux remaining after Li has replaced Na in the interfibre fluid. The estimates are not very accurate, chiefly because the extrapolations are based on rather few points. However, there were two reasons for not using more points. First, prolonged exposure to Na-free media causes appreciable changes in the concentration of sodium within the fibres. Secondly, we often wished to observe the effects of a number of different solutions on the same muscle, so that the muscle could serve as its own control. The specific activity of the ^{24}Na samples and the rate of turnover of labelled sodium in frog muscle fibres were such that the period spent in each solution had to be kept to a minimum.

The validity of such extrapolations is supported by the fact that, during the second exposure to Na-free medium in Fig. 2, the linearity of the decline in the logarithm of ^{24}Na efflux was maintained, within the accuracy of the counts, for well over 2 hr. Similar behaviour was observed in four other experiments (one with choline substitution, and three with lithium). Since no counts taken during the first 40 min after removing the muscles from ^{24}Na were used for the extrapolations, it was also legitimate to treat the decline in log. efflux in normal Ringer's solution as perfectly linear over periods of less than an hour; over longer intervals there was perceptible flattening of the curves but this was not sufficiently marked to introduce serious errors.

On three successive exposures to Na-free Ringer's solution the efflux from the muscle of Fig. 2 was estimated to have decreased to 0·46, 0·48 and 0·42 of the control efflux, thus showing fair constancy in the magnitude of the response over a period of about 6 hr. In thirty consecutive experiments in which the response was measured within 5 hr of dissecting the muscle, the Na efflux promptly decreased to an average of 0·475 (S.D. = ±0·047) of its initial value when the muscle was transferred to Li Ringer's solution.

A small but apparently consistent difference was observed between the response in Li Ringer's solution and that in choline Ringer's solution. The reduction in efflux averaged 10% less (2–16% in 5 muscles) in choline than in lithium. Within 15 sec of transferring a sartorius muscle to choline Ringer's solution, fibrillation of the muscle begins and lasts for up to a minute. This is presumably due to a pharmacological action of choline at the neuromuscular junction, since it can be blocked by $2{\cdot}5\times10^{-5}$ M tubocurarine. Neither fibrillation nor spontaneous contraction occur when a muscle is put into lithium Ringer's solution. However, the slight difference in the extent of the reduction in Na efflux between choline and lithium did not seem to be due to the occurrence of fibrillation in one case and not in the other, since it persisted when tubocurarine was added to the medium before substituting choline for sodium. We have not explained nor further investigated the difference in behaviour of the muscle between choline and lithium, and since there was no fibrillation in lithium it was used for the rest of the experiments described in this paper.

The effect of Na-free medium on other amphibian muscles

The prompt reduction in Na efflux in lithium Ringer's solution is observed in several muscles of *R. temporaria* that have been tested, including both those in which the slow-fibre system is well represented (in *R. pipiens*; Kuffler & Vaughan Williams, 1953), such as *a*, *b*, *c* and *d* in Fig. 3, and those in which it is not (*e* and *f* in Fig. 3). It is also observed in the sartorius of another species, *R. pipiens* (Fig. 3*h*), and of a member of another family, *Bufo vulgaris* (Fig. 3*g*).

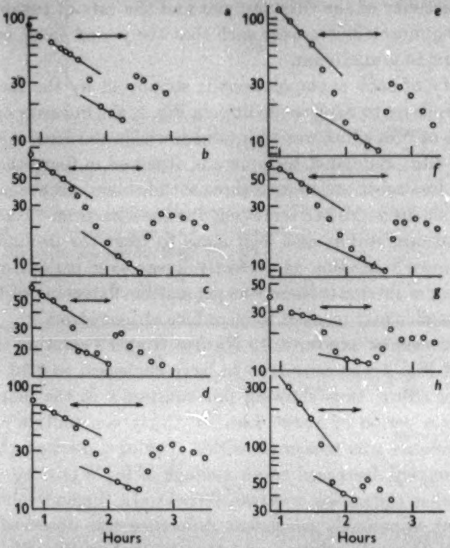

Fig. 3. The effect of lithium, applied during the arrows, on the efflux of ^{24}Na from various amphibian muscles. Ordinates, effluxes of ^{24}Na (counts/min^2), log. scales; abscissae, time. *a–f*, muscles from *Rana temporaria*: *a*, biceps; *b*, semimembranosus; *c*, extensor longus dig. IV; *d*, semitendinosus; *e*, gracilis; *f*, peroneus; *g*, sartorius from *Bufo vulgaris*; *h*, sartorius from *Rana pipiens*.

The magnitude of the reduction in efflux was apparently similar in all these muscles, although the appreciable size of some of them prevented accurate measurement of the effect. Their weights ranged from 5 to 106 mg, and there was a correspondingly wide variation in the ratios of weight to surface area and in their maximum thicknesses. The delay in the reduction of the Na efflux after transfer to the Na-free medium was roughly correlated with the thickness of the muscles, and probably arose from the time required for Li to replace Na in the interfibre spaces.

Variation in the rate constants for Na efflux with internal Na concentration

It will be noticed in Fig. 2 that when the logarithm of the efflux of labelled Na (written as M_0^* and expressed in counts/min^2) is plotted against time, the reduction in efflux on transferring the muscle to lithium is not accompanied by an obvious change in the slope of the graph. This slope, whose value is $(1/M_0^*)\ (\mathrm{d}M_0^*/\mathrm{d}t)$, may be termed the instantaneous rate constant for decline of Na* (that is, labelled Na) efflux; it will be written as k_1' in normal Ringer's solution and k_2' in lithium. The observation that k_1' and k_2' were approximately equal was somewhat unexpected, since the simplest prediction would be that if the efflux were abruptly halved, as it is in lithium, the rate constant for its decline would also be halved. This expectation follows from the assumptions that the muscle behaves as a single-compartment system in which the internal Na is homogeneously distributed and the total efflux of Na, M_0, is directly proportional to the total internal Na concentration, i.e. that

$$M_0 = p.[\mathrm{Na}] \tag{1}$$

where p is a constant, and that the membrane cannot discriminate between labelled and unlabelled Na, i.e. that

$$\frac{M_0^*}{M_0} = \frac{[\mathrm{Na}^*]}{[\mathrm{Na}]}. \tag{2}$$

For since by definition

$$\frac{\mathrm{d}[\mathrm{Na}^*]}{\mathrm{d}t} = -M_0^* \tag{3}$$

it may be seen by combining (1) and (2), differentiating, and substituting from (3), that

$$k_1' = \frac{1}{M_0^*} \cdot \frac{\mathrm{d}M_0^*}{\mathrm{d}t} = -p. \tag{4}$$

(Note that these equations are only valid when M_0^* is expressed in counts/min^2 and [Na*] in counts/min; if the units used are instead moles/cm^2.sec and moles/l., it is necessary to introduce the surface : volume ratio of the fibres into equation (3), so that $k_1' = -p(A/V)$ and p has the dimensions cm/min). If now the reduction of efflux in lithium represents an alteration of the proportionality constant to p', then

$$k_2' = -p' \tag{5}$$

whence k_1'/k_2' should be equal to p/p', and should have a value of rather over two.

Table 2 gives values for k'_1, k'_2 and the similar rate constants for decline of total radioactivity ($= (1/[\mathrm{Na}^*])\,(\mathrm{d}\,[\mathrm{Na}^*]/\mathrm{d}t)$), k_1 in normal Ringer's solution and k_2 in lithium, in the experiment of Fig. 2 and in four similar experiments in which exposure to Li Ringer's solution was maintained for two or more hours. None of the k's are strictly constant over long periods, and k'_1 and k_1 were therefore calculated from the counts taken immediately before transferring to lithium, while k'_2 and k_2 refer to the interval from 20 to 60 min after transferring to lithium. Since there was no instantaneous change in [Na*] on going into lithium, it follows from equation (3) that the ratio k_1/k_2 must be equal to the ratio of the Na* effluxes immediately before and after transferring to lithium, whether the assumption in equation (1) is true or not. The average value of k_1/k_2 was 3·10, which was, in fact, rather higher than the average flux ratio of 2·1 for other experiments discussed above (see p. 595); the difference probably arises because in the experiments of Table 2 the values of k_2 were calculated after a somewhat longer exposure to lithium than in the procedure used in the measurement of the flux ratios. The discrepancy that gave rise to this enquiry, was then, that the ratio k'_1/k'_2 was close to unity, instead of being the same as the flux ratio, in other words equal to k_1/k_2. It will be seen from Table 2 that, with one exception, k'_1/k'_2 was only a little greater than 1. The same tendency was observed in almost every other experiment (see, for example, Figs. 1, 2, 3, etc.), though the periods in lithium were often too short for accurate values of k'_2 to be worked out. It can only be concluded that the prediction made in the preceding paragraph must be based on an incorrect assumption.

TABLE 2. Values of the rate constants for decline of ^{24}Na efflux (k'_1, normal; k'_2 in Li) and for decline of total radioactivity (k_1, normal; k_2, in Li), and of their ratios. k'_1 and k_1 were calculated from counts taken in normal Ringer's solution just before transferring to Li Ringer; k'_2 and k_2 were calculated from counts taken during the period 20–60 min after transferring to Li Ringer's solution

Expt. no.	Rate constants (hr^{-1})				Ratios			
	k'_1	k'_2	k_1	k_2	$k'_1:k'_2$	$k_1:k_2$	$k'_1:k_1$	$k'_2:k_2$
1	0·59	0·56	0·58	0·26	1·05	2·23	1·02	2·15
2	1·26	0·60	1·13	0·23	2·10	4·91	1·11	2·61
3	0·74	0·67	0·68	0·32	1·10	2·12	1·09	2·09
4	1·04	0·82	0·99	0·29	1·27	3·41	1·05	2·82
5	1·22	1·04	1·33	0·47	1·17	2·83	0·92	2·21
Mean	0·97	0·74	0·94	0·31	1·34	3·10	1·04	2·38

Examination of the relative sizes of k'_1 and k_1, showed that they were always very nearly equal. This was, however, to be expected, since analyses of the total Na content of muscles suspended in normal Ringer's solution under conditions similar to those in the tracer experiments showed that the net rate of gain of Na was slow in comparison with the turnover of Na*, so that the muscles were effectively in a steady state. It will be obvious on referring to equation (2) that if both M_0 and [Na] are constant, then k_1' must be equal to k_1, whatever the relation between M_0 and [Na] when [Na] varies. The average of 1·04 for k_1'/k_1 shown in Table 2 (and similar values in a number of other experiments) was therefore satisfactory, but provided no test of the applicability of equation (1). Investigation of the similar ratio, k_2'/k_2, for muscles in lithium, does on the other hand throw light on this question, since in a sodium-free medium the muscles cannot be in a steady state if there is any observable efflux of sodium. If equation (1) is valid for muscles in lithium, then it follows directly from differentiating it that the rate constants for decline of Na efflux and for decline of total internal Na should again be equal, i.e. that $k_2 = k_2'$. The observation that the average value of k_2'/k_2 was actually 2·38 is an alternative way of showing that a false assumption must have been made.

While the muscles were in lithium the sodium influx was zero, so that the specific activity of the labelled Na in them remained constant, and [Na*] could be equated with [Na]. By plotting Na* efflux against [Na*] it was therefore possible to examine directly the way in which the efflux varied as the internal Na concentration fell. When this was done, the reason for the inequality of k_2' and k_2 at once became clear, for although the relationship between M_0^* and [Na*] was quite accurately linear during the first two hours in lithium (cf. the upper parts of the curves shown in Fig. 4*B* and *C*), the points did not lie on a straight line passing through the origin, as equation (1) would demand, but on a line given by

$$M_0^* = a([\mathrm{Na}^*]-b). \tag{6}$$

The intercept *b*, expressed as a fraction of the total counts/min in the muscle at the moment of transference to lithium (i.e. as a fraction of the total intracellular [Na] in the muscle), had an average value of 0·54 in Expts. 1–4 of Table 2. It will be seen that differentiation of equation (6) leads to the relation

$$k_2 = \left(1-\frac{b}{[\mathrm{Na}^*]}\right)k_2' \tag{7}$$

which fits reasonably well with the figure quoted above for k_2'/k_2. In lithium Ringer's solution, the Na efflux thus seemed to be behaving as if it were proportional to only part of the Na content of the muscle, the remainder of the Na being inexchangeable under these conditions.

The two experiments illustrated in Fig. 4*A* had the object of examining the efflux versus internal [Na] relationship over a greater range. In one case a sartorius muscle was exposed to Li Ringer for over 6 hr, and in the other a gracilis minor muscle spent about 3 hr in Li Ringer. It will be seen in *B* and *C* that although at first the Na* efflux declined more or less linearly with [Na*], the plots eventually curved round towards the origin. The simple concept of an inexchangeable fraction accounting for over half the internal Na is therefore ruled out, but it would in any case have been incompatible with the results of

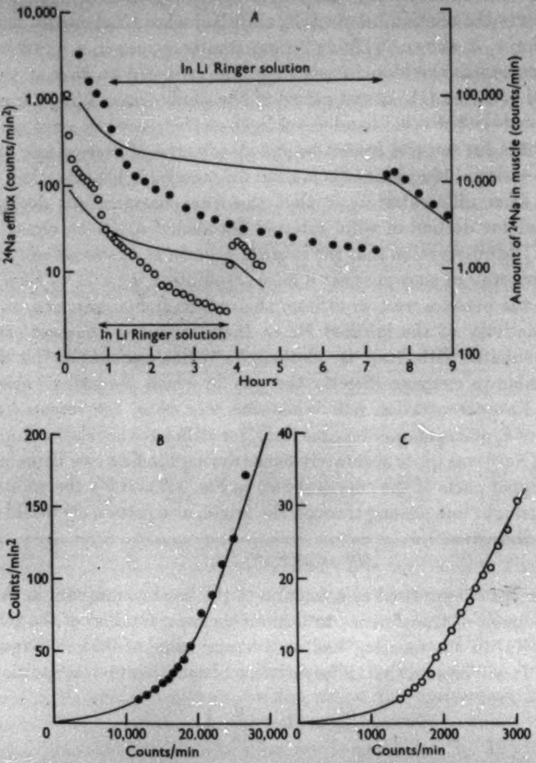

Fig. 4. Two experiments to examine the changes in ^{24}Na efflux during a long period of exposure to Li Ringer's solution. In *A* the effluxes are plotted on a logarithmic scale against time. ●, sartorius muscle, weight 40·7 mg, from *Rana temporaria*, temperature 19° C; ○, gracilis minor muscle, weight 28·9 mg, from *R. pipiens*, temperature 19° C. The lines show the corresponding decline in the total radioactivity of the muscles (right-hand scale). *B* and *C* show the efflux values in Li Ringer plotted against total radioactivity. In both cases the first two points after transferring to Li have been omitted, as have a few other points (to avoid over-crowding). The curves in *B* and *C* were worked out from eqns. 9 and 10 using the following values:

B	*C*
$[\mathrm{Na}_1^*]_0 = 6440$ counts/min	$[\mathrm{Na}_1^*]_0 = 1205$ counts/min
$[\mathrm{Na}_2^*]_0 = 18{,}760$ counts/min	$[\mathrm{Na}_2^*]_0 = 1735$ counts/min
$g = 0{\cdot}95\ \mathrm{hr}^{-1}$	$g = 1{\cdot}25\ \mathrm{hr}^{-1}$
$h = 0{\cdot}08\ \mathrm{hr}^{-1}$	$h = 0{\cdot}10\ \mathrm{hr}^{-1}$

analysing muscles soaked for long periods in Li Ringer (see Keynes & Swan, 1959), since the amount of Na which is so firmly held as to be genuinely irreplaceable by Li does not seem to be greater than 1 or 2 m-mole/kg. An alternative explanation for the curves in Fig. 4*B* and *C* would be that the Na in the muscle is located in two separate compartments, whose contents exchange with external ions independently and *in parallel*. If in normal Ringer's solution the two compartments had equal rate constants (k_1) for loss of Na*; if in Li the efflux from one compartment were initially unaltered, while the efflux from the other were cut down by a factor of 10–15; and if the efflux from each compartment were proportional to the [Na] in that compartment, then the Na* efflux in lithium would be given by

$$M_0^* = g[\mathrm{Na}_1^*] + h[\mathrm{Na}_2^*], \tag{8}$$

where g was roughly equal to k_1 and h was 10–15 times smaller. Both $[\mathrm{Na}_1^*]$ and $[\mathrm{Na}_2^*]$ would decrease exponentially, with rate constants g and h respectively, and if their initial values were $[\mathrm{Na}_1^*]_0$ and $[\mathrm{Na}_2^*]_0$ we should have

$$[\mathrm{Na}^*] = [\mathrm{Na}_1^*]_0 . \mathrm{e}^{-gt} + [\mathrm{Na}_2^*]_0 . \mathrm{e}^{-ht} \tag{9}$$

and

$$M_0^* = g . [\mathrm{Na}_1^*]_0 . \mathrm{e}^{-gt} + h . [\mathrm{Na}_2^*]_0 . \mathrm{e}^{-ht}. \tag{10}$$

The smooth curves drawn in Fig. 4*B* and *C* are the result of fitting the experimental points by trial and error to a system of this sort.

This 'two-compartment' hypothesis satisfies the further criterion of predicting the very large and virtually immediate increase in efflux on replacing the muscles in sodium Ringer's solution, which was one of the most striking features of experiments in which muscles were subjected to long exposures to lithium, because the Li-sensitive fraction would be 'unmasked' by the total depletion of the radioactivity in the Li-insensitive fraction. It is also consistent with the observed tendency for the value of k_1 when the muscles were finally returned to ordinary Ringer's solution to be rather close to its value for the period preceding exposure to lithium. However, quantitative arguments to support these assertions will not be presented, since although the hypothesis seemed to us for some time to provide a rather attractive explanation for the observations in Fig. 4, we were finally compelled to abandon it. The reasons for its rejection are discussed on pp. 619–20.

The effect on Na efflux of external Na concentrations between 0 *and* 111 mM

The experiments described so far involved the complete replacement of the Na in the external medium. It was of interest to see what would happen if the Na were only partly replaced by Li, and Fig. 5 shows how the magnitude of the reduction in Na efflux varied with external [Na]. In each of these experiments, one or two measurements of the reduction in Na efflux at intermediate Na concentrations were bracketed by measurements in the Na-free medium.

The results were accepted only if the sizes of the reductions in efflux for 0 mM-Na during the later stages of the experiment were within 8% of the mean response for that particular muscle. To obtain measurements at three intermediate Na concentrations it was necessary to perform an experiment lasting more than 12 hr after completion of the dissection. The desired stability in the response to the Na-free medium was not obtained until the external K concentration had been raised to 10 mM by adding extra KCl to the solutions. In the experiments of Fig. 5, 10 mM-K was present throughout, and all the results satisfied the criterion stated above. In other tests it was verified that this change in external [K] had little or no effect on the extent of the response to Na-free solutions. The results in Fig. 5 were normalized to a reduction in Na efflux in 0 mM-Na to 47·5%, which was the mean response in a large series of muscles and also happened to be the mean response for these four muscles.

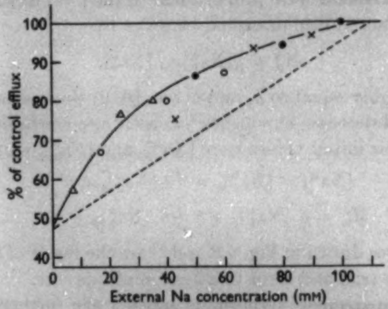

Fig. 5. Relative magnitude of ^{24}Na efflux when external [Na] is varied between 0 and 111·2 mM by substituting Li. △, expt. 71; ○, expt. 72; ×, expt. 73, ●, expt. 88

The size of the component of the Na efflux which appears to depend on the presence of Na in the external medium rises hyperbolically with external Na concentration. Using the method of Lineweaver & Burk (1934) an equation of the Michaelis-Menten type was fitted to the results in Fig. 5, giving a half-saturation value of 38 m-mole external Na/l., and only reaching saturation at about 130 mM—a concentration well above the normal level of [Na]. However, the relationship was not examined experimentally at external Na concentrations above 111 mM. On the basis of some other observations, the relationship seemed to be rather similar when there was only 2·5 mM-K in the solutions, and when choline was used instead of Li as a sodium substitute.

The results in Fig. 5 help to explain the difference between the rate at which the Na efflux approaches its new value on removal of Na and the rate at which it recovers on restoring external Na. As may be seen in many of the figures in this paper, the recovery is much the faster process.

Calculations showed that a combination of Fig. 5 with an exponential exchange of interfibre Na having a half-time of 2 min (as observed by Levi & Ussing (1948), and roughly verified in a number of our own experiments) gave theoretical curves which closely resembled the observed time courses of the changes in Na efflux on removal and restoration of external Na.

When a sartorius muscle is equilibrated in a sodium-free solution, the interfibre fluid can never be wholly devoid of Na, even if the muscle is a small one, for as long as there is an efflux of Na from the fibres there must be a gradient of [Na] through the extracellular space. Using the appropriate equation given by Hill (1928) for a flat muscle exposed to the medium on both sides, taking the diffusion coefficient in the interfibre space as $5{\cdot}6 \times 10^{-6}$ cm^2/sec (Keynes, 1954), the muscle thickness as 0·6 mm, the internal [Na] as 8·3 m-mole/l. fibre and the rate constant for Na extrusion as 0·9 hr^{-1} (from Table 4), it can be calculated that after transferring the muscle to Na-free Ringer's solution the interfibre [Na] at its centre will not for some time fall much below 1 mM. Since the calculated half-saturation value in Fig. 5 is much larger than this, an external accumulation of Na is unlikely to have caused appreciable errors in the results, even at 0 mM-Na.

Summation of Na-dependent and K-dependent components of Na efflux

It has been shown by Keynes (1954) that the Na efflux in frog muscle varies appreciably with external K concentration in the range 0–10 mM-K. The effect was more obvious in a very small muscle than in a large one, possibly because the slowness of extracellular diffusion makes it impossible to achieve really K-free conditions at the centre of the muscle (cf. calculation for interfibre [Na] above). In the small sartorii used in the present experiments the reduction in Na efflux on lowering interfibre [K] showed up fairly well, and could be demonstrated to give a partial summation with the reduction in Na efflux on removal of external Na. In the experiment illustrated in Fig. 6, a small amount of Na phosphate buffer was present throughout (Solution *B*, Table 1) and when the external [Na] was reduced from 113·7 to 2·5 mM the Na efflux fell to 0·61 of its control value; this effect was reproduced 2·5 hr later. In the interval, the response to transferring the muscle to a K-free medium containing 2·5 mM-Na was a reduction in the efflux to 0·44 of the control. This was an appreciably larger effect than was finally obtained when only K was omitted from the medium (a reduction to 0·71). Summation was not complete, as it presumably would have been if the effects of removing Na and K had been exerted on entirely independent components of the Na efflux; in a number of experiments the reduction in efflux in 0 mM-K, low-Na averaged 85% of the sum of the separate responses. In Fig. 6 the responses could be said to have multiplied rather than adding, since $0{\cdot}71 \times 0{\cdot}62 = 0{\cdot}44$; but this may have been a coincidence.

Similar experiments in which the changes of solution were made in different sequences all showed some summation of the Na and K effects. Summation was also observed in K-free and Na-free media which were unbuffered and neutralized to about pH 7·6. And a further drop in Na efflux could be demonstrated when a muscle was transferred directly from Li Ringer's solution with normal [K] to K-free Li Ringer.

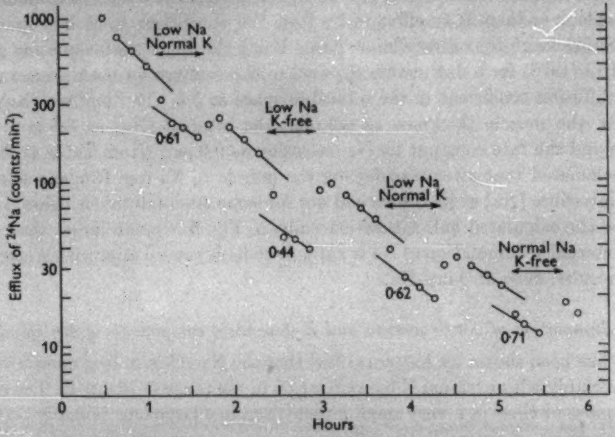

Fig. 6. Summation of the reduction in ^{24}Na efflux on removing almost all the external Na (leaving only 2·5 mM-Na) with the reduction in a K-free medium. Sartorius, weight 28·3 mg. Temperature 18·8° C. Semi-log. scale.

Change with time in vitro of the relative magnitudes of the two components of the Na efflux

While the magnitude of the prompt reduction in Na efflux on removal of external Na remains constant for several hours after dissection when the muscle is in a medium containing 2·5 mM-K (see Fig. 2), the response eventually declines and is no longer demonstrable 12–24 hr after dissection. Meanwhile the magnitude of the K effect increases with time. Figure 7 shows the results of an experiment on two sartorius muscles dissected from the same frog at approximately the same time. One muscle (upper curve) was then equilibrated with radioactive Ringer's solution and the efflux of ^{24}Na measured after transfer to inactive media. The reductions in efflux in Na- and K-free media were calculated as 0·39 and 0·81, respectively. The second muscle (lower curve) was kept in well stirred normal Ringer's solution at room temperature for 20 hr after dissection, and then equilibrated for the same period in radioactive Ringer's solution. When the changes in Na efflux were subsequently investigated, the Na-free solution no longer had any effect, but the

K-free solution had an appreciably larger effect than on the first muscle. The same reciprocal change in the relative sizes of the responses could be shown in a single muscle, by first testing it as in the upper curve of Fig. 7, then storing it for 20 hr in normal Ringer's solution at room temperature, re-equilibrating it in a radioactive solution, and finally testing again. Invariably the response to the Na-free medium disappeared, and the response to the K-free medium showed an increase.

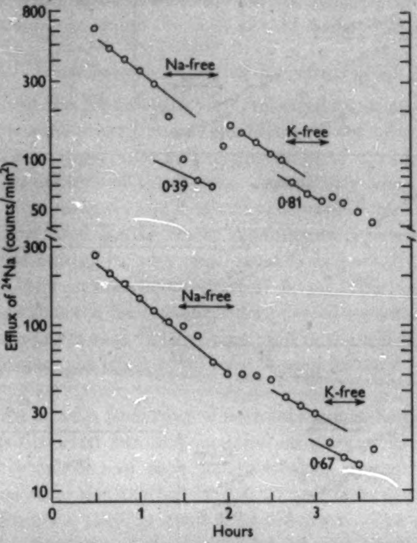

Fig. 7. Reciprocal changes in the responses to Na-free and K-free solutions for paired muscles stored in normal Ringer's solution for different periods. Ordinate (both curves), efflux of ^{24}Na (counts/min^2); abscissa (upper curve), time in hours, zero 3·5 hr after dissecting muscle; abscissa (lower curve), time in hours, zero 23·5 hr after dissecting muscle. Sartorii, weight 44·7 mg (upper), 44·8 mg (lower). Temperature 19·7° C (upper), 19·5° C (lower). Semi-log. scale.

When the medium in which the muscle was stored for 20 hr contained 0·011 M glucose and was stirred by bubbling pure oxygen into it, the change in behaviour seen in the lower curve of Fig. 7 still took place. This suggests that the disappearance of the Na-free effect does not arise from an inadequacy in the supply of glucose and oxygen to the muscle.

The reciprocal changes in the responses could be partly reversed by prolonged exposure of the muscle to the Na-free medium. The upper curve of Fig. 8 shows the reduction in Na efflux to 0·49 and 0·82 when a muscle was transferred, 6 and 7·5 hr after equilibration with ^{24}Na, to Na- and K-free solutions, respectively. The muscle was then soaked for about 6 hr in well stirred normal Ringer's solution, re-equilibrated with ^{24}Na, and the responses to removal of Na and K retested. There was now no reduction in efflux on removal of external Na, but a reduction to 0·67 on removal of K. The muscle was next left in Li Ringer's solution for 3 hr, during which time the total internal [Na] is estimated to have decreased by 37 %. A final test then showed

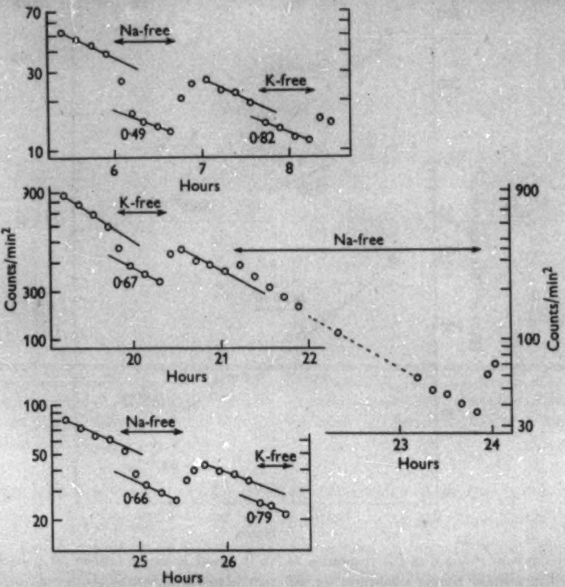

Fig. 8. Reversal of reciprocal changes in the responses to Na-free and K-free solutions by long exposure to Li Ringer's solution. Sartorius, weight 28·2 mg, temperature 16·6° C; zero time was 3 hr after dissecting muscle. Semi-log. scale.

a reduction in efflux to 0·66 in the absence of Na, and to 0·79 in the absence of K. At this time, 29 and 30 hr after dissection, the sizes of the responses had thus been restored towards those typically seen within a few hours of dissection.

In other experiments the reduction in Na efflux in Li Ringer's solution, after remaining approximately constant for several hours, was observed to decline progressively over a period of a few hours, and even to be replaced (as in Fig. 8) by a transient increase. It may therefore be asked whether replacement of interfibre Na with Li *in vivo* or very soon after dissection would reduce the efflux by much more than the usual 50%. We tried to answer this question by equilibrating a muscle with ^{24}Na for 10 min, 30 min after pithing the frog, and examining the Li effect 1 hr later. The Na efflux was reduced to 0·45 of the control value, so that the response was close to the average value of 0·475 observed 4·5 hr after dissection. It would be difficult to make the necessary measurements much more quickly, and it seems reasonable to conclude from this result, and from the observed constancy of the response for the next few hours after dissection, that the response of a really fresh muscle would probably be much the same as that 4·5 hr after dissection.

Rate of change in vitro of Na and K contents of muscles

The restoration of the Na-free effect by prolonged exposure to Li Ringer's solution, and some similar experiments at different temperatures (see below), suggested that the disappearance and reappearance of the effect might be a function of the Na content of the muscle. Accordingly some analyses were done to see what changes in Na and K content occurred in muscles treated in a similar way to those used for the tracer experiments. Two series of muscles were examined, these being (*a*) very small muscles with weights ranging from 19 to 29 mg, and (*b*) larger muscles weighing 47–58 mg. This was done because there was evidence that the Na-free effect disappeared sooner after dissection in small sartorius muscles than in large ones; and in the still smaller extensor longus dig. IV muscles the effect could only be seen for very few hours after dissection.

Pairs of sartorius muscles from frogs of both sexes which had spent 7–9 days in captivity were dissected free and mounted on glass racks as in the ^{24}Na experiments. One member of each pair was then trimmed, blotted and weighed, while the other was exposed for 3, 6, 12 and 24 hr at 19–21° C to well stirred normal Ringer's solution containing 2·5 mM-K. The second muscle was then trimmed, blotted and weighed, and both were analysed for Na and K. The concentrations of Na and K in the fibre water were calculated on the assumptions that the interfibre fluid accounted for 13% of the wet weight (Boyle & Conway, 1941), and that this fraction did not change appreciably during 24 hr exposure to an artificial medium (Desmedt, 1953). The results are summarized in Table 3.

In the four pairs of small muscles the average initial Na concentration in the fibre water was 13 mM; it increased at an average rate of 1·19 mM/hr (S.E. ± 0·14 mM/hr). The mean initial K concentration was 148 mM, and in each comparison the K content decreased by somewhat more than the rise in Na. The larger muscles started with almost the same Na and K contents (14 and 145 mM), but gained Na more slowly (0·66 ± 0·06 mM/hr). The difference in rate of gain of Na was statistically significant ($P < 0·02$). A progressive loss of weight with time was observed in each group of muscles, averaging 0·8%/hr in the small muscles and 0·5%/hr in the large ones. Two further series of analyses, which will not be presented in detail, gave rather more scattered results, possibly because the muscles were taken from a less homogeneous group of frogs, and temperature was less closely controlled; however, they showed a similar difference in the average rate of gain of Na between large and small muscles.

TABLE 3. Changes in Na and K contents of muscles with time after dissection, in normal Ringer's solution containing 2·5 mM-K, at 19–21° C. The first column gives the initial weight (of the control muscle) and the final weight (of the test muscle). See text for assumptions made in calculating Na concentrations in fibre water

Wet weights of muscles (mg)	Time from dissection (hr)	[Na] in control (mM)	Change in [Na] (mM)	[K] in control (mM)	Change in [K] (mM)
		(a) Muscles weighing 19–29 mg			
28·7, 28·0	3	13	+ 3	148	− 9
25·0, 23·1	6	13	+ 9	142	−15
19·2, 17·8	12	13	+16	157	−30
22·6, 20·6	24	11	+22	143	−28
Mean	—	13	—	148	—
		(b) Muscles weighing 47–58 mg			
57·9, 56·5	3	17	+ 2	140	− 4
46·5, 44·2	6	11	+ 3	147	− 8
47·5, 43·7	12	16	+ 8	150	− 8
48·4, 44·9	24	10	+19	144	−25
Mean	—	14	—	145	—

The difference in behaviour between large and small muscles could be related to a difference in mean fibre diameter; the natural length of the 50 mg sartorius muscles was only about 25% greater than that of the 25 mg muscles, so that if they contained an equal number of fibres the average diameter ratio would be about 1·6:1. Another factor which will influence the ability of muscles *in vitro* to retain their K and keep down their Na is the slowness of diffusion in the interfibre spaces; the mean extracellular concentration of K and of any other substances leaking out of the muscles will be higher in large than in small muscles.

Our ^{24}Na experiments were not designed specifically to determine how soon after dissection the effect on the Na efflux of Na-free solutions disappeared. However, we gained the impression that the effect persisted for about 12 hr in 50 mg muscles, and for a somewhat shorter period in smaller muscles. If an excessive Na content of the fibres is indeed the factor responsible for abolishing the effect of Na-free solutions, then the values in Table 3 suggest that the critical concentration of Na in the fibre water is in the neighbourhood of 20 mM.

The effect of temperature on the Na efflux in Na- and K-free media

When the effect of cooling on the relative sizes of the Na-free and K-free effects was investigated, we were surprised to find that at 1° C the Na efflux was apparently unaltered by exposure of the muscle to Li Ringer's solution. The upper half of Fig. 9 shows the typical reduction in Na efflux when a sartorius was transferred to Li Ringer's solution at room temperature (19·8° C); the response was reproduced 4 hr later. During the intervening period the muscle was transferred to normal Ringer's solution at 1° C, as a result of which the Na efflux was reduced to 0·24 of its value at room temperature ($Q_{10} = 2{\cdot}15$). When, however, the muscle was exposed to Li Ringer's solution also at 1° C, no further reduction in Na efflux was seen.

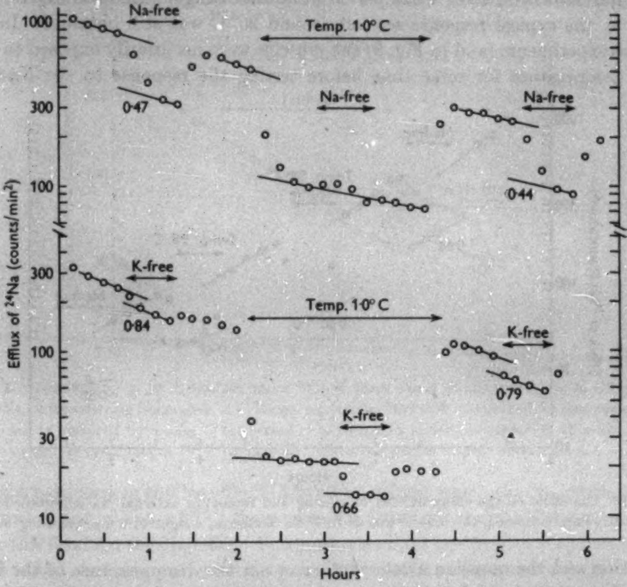

Fig. 9. Upper curve: after a muscle had been kept at 1° C for about 40 min, the efflux of ^{24}Na was no longer reduced in Li Ringer's solution. Sartorius, weight 54·9 mg, room temperature 19·8° C. Lower curve: on similar treatment, a muscle from another frog gave a relatively larger K-free effect at the low temperature. Sartorius, weight 26·4 mg, room temperature 18·9° C. Semi-log. scale.

The lower part of Fig. 9 shows a similar experiment designed to compare the change in Na efflux caused by a K-free medium at room temperature and at

1° C. At 18·9° C the K-free response was to 0·84 and 0·79 of the control value. At 1° C the Na efflux in normal Ringer's solution fell to 0·19 ($Q_{10} = 2{\cdot}55$), and the K-free medium caused a further reduction in efflux to 0·66 of the control value at 1° C. In contrast to the abolition of the Na-free effect at the low temperature, there was thus an increase in the relative magnitude of the K-free effect.

In a number of similar experiments the effect of the Na-free medium was found to have disappeared after the muscle had been at 5 or 10° C for 40–60 min. However, after a like period in normal Ringer's solution at 14° C or above, the typical response seen at around 20° C was still obtained. In all these experiments (and in Fig. 9) the muscles were necessarily exposed to the low temperature for some time before testing the response to the Na-free solution and the question arose whether or not the disappearance of the Na-free response occurred immediately the temperature was lowered. This was answered by experiments of the kind illustrated in Fig. 10, in which temperature and external [Na] were changed simultaneously. It will be seen that in this muscle the response to Li Ringer's solution at 19·7° C was a reduction in efflux to 0·44 at the beginning and to 0·50 at the end of the experiment. A temperature change to 9·8° C in normal Ringer's solution reduced the efflux to 0·47 ($Q_{10} = 2{\cdot}15$): but the same temperature change in Li Ringer's solution produced a reduction to 0·30. Hence the response with both variables altered simultaneously was appreciably greater than when either was changed separately. The tentative conclusion is that the Na-dependent component of the Na efflux is not eliminated by low temperature *per se*, but disappears as a

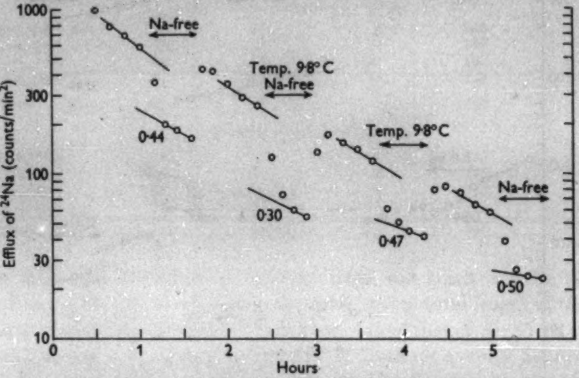

Fig. 10. The effect on the efflux of ^{24}Na of cooling and removing external Na simultaneously. Except where indicated, the muscle was at 19·7° C. Sartorius, weight 41·9 mg. Semi-log. scale.

consequence of changes in the muscle which take place when the muscle is cooled for 40 min or more.

It seemed possible that the change in question might be a rise in the Na content of the muscle brought about by cooling. This suggestion was supported by the experiment illustrated in Fig. 11. After equilibration in radioactive Ringer's solution, a sartorius muscle was transferred to inactive normal Ringer's solution at 10·0° C, this temperature being maintained throughout the rest of the experiment. After 70 min at the low temperature, the muscle was transferred to Li Ringer's solution, still at 10·0 °C; there was, as expected, no prompt reduction in the Na efflux. After 2 hr, during which the Na content of the muscle fell by about 35 %, the muscle was returned to normal Ringer's solution; this doubled the Na efflux. Forty minutes later the Na-free response was again tested, and was now found to be nearly normal, a reduction in the efflux to 0·61 being observed. Thus prolonged exposure to Li Ringer's solution, whose most obvious consequence would be a drop in the Na content of the muscle, has the effect of restoring the response.

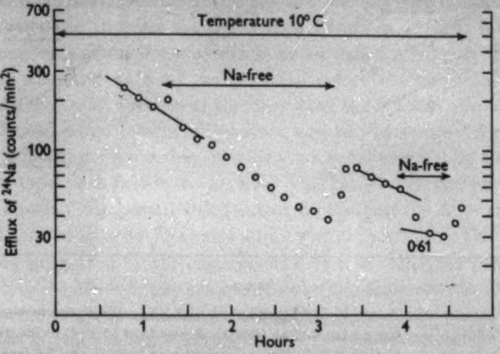

Fig. 11. After cooling to 10° C for just under 70 min, there was a transient increase in efflux of ^{24}Na on transferring the muscle to Li Ringer's solution. However, the normal Na-free response could be restored by a long (2 hr) period of exposure to Li Ringer's solution although the temperature remained at 10° C throughout. Sartorius, weight 37 mg. Semi-log scale.

A final example of the effect of low temperature in abolishing the Na-free response, and of its restoration by soaking in Li Ringer's solution for some time, is given in Fig. 12. Muscles *A* and *B* were dissected from the same frog, and equilibrated with ^{24}Na Ringer's solution at and for the same time under identical conditions. Muscle *A* was then transferred to inactive normal Ringer's solution at 19·8° C, while muscle *B* was kept at 2° C for 3 hr in a phosphate-buffered inactive solution containing 0·5 mM-K. When subsequently the Na-free responses of the two muscles were examined at 19·8° C, muscle *A* gave a normal response (to 0·52), but muscle *B* gave no reduction of efflux in Li Ringer's solution. However, after 1·5 hr in the Na-free medium, a return of muscle *B* to normal Ringer's solution more than doubled the efflux, and when the muscle was finally tested again in Li Ringer's solution the efflux was reduced to 0·50. Thus the long period in Li, during which the Na content of the muscle dropped 43%, restored the Na-free response virtually to normal.

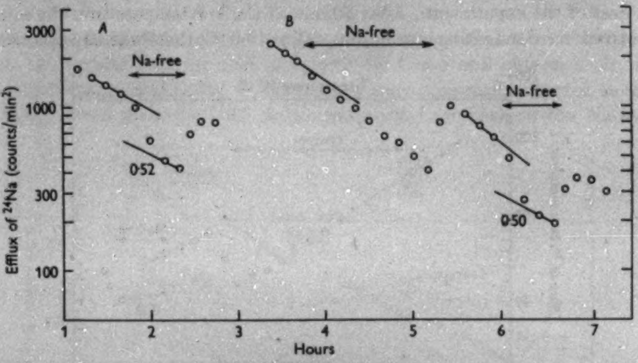

Fig. 12. Two muscles were dissected and equilibrated with ^{24}Na. While the response of *A* to Li Ringer's solution was being observed at 19·8° C, *B* was kept at 2° C in inactive low-K Na Ringer's solution. On testing *B* in Li Ringer's solution at 19·8° C, the Na-free response had disappeared, but it was restored by exposing to Li Ringer's solution for 1·5 hr. Weight of muscle *A* was 42·6 mg. Weight of muscle *B* was 42·4 mg. Semi-log. scale.

Attempts to check the supposition that the disappearance of the Na-free effect at low temperatures arises from an increase in the Na content of the muscle have not given entirely satisfactory results, since two alternative methods of estimating the rise in internal [Na] do not agree very well. The Q_{10} for the influx of labelled Na is of the order of 1·2 between 20 and 1° C (unpublished observations by E. Flückiger and R. D. Keynes on extensor longus dig. IV muscles gave a mean value of 1·23; we have checked this figure using gracilis minor muscles, for which two measurements gave a mean of 1·25), whereas the Q_{10} for Na efflux in the experiments discussed in this paper averaged 2·3 (in ext. longus dig. IV muscles the average was 2·25, in gracilis minor 2·5). Taking the influx and efflux at room temperature to be equal at 4·2 pmole/cm^2.sec (see Table 4), it can be calculated from the tracer data that on cooling muscles to 1° C, Na should initially be gained at a rate of roughly 5 m-mole/l. fibre water per hour, since the influx is reduced less than the efflux. However, direct determinations of the Na in pairs of muscles where one member of each pair was trimmed, blotted and weighed soon after dissection, while the other was kept at 0·6° C for 2·2 hr before being taken for analysis, failed to reveal a change in Na content as large as this. Thus in thirteen pairs of muscles the estimated change in internal [Na] was only +2·1 m-mole/l. fibre water (S.E. ±0·8 mM). At the same time there was an appreciable loss of K (−8·4 ± 1·2 mM), and the muscles gained both in weight (+3·6 ± 0·8%) and in water content (+1·0 ± 0·3%), suggesting that the net entry of Na into the fibres predicted from the tracer results was probably taking place, but was being obscured by a simultaneous swelling of the fibres. Even a rather small reduction in the extracellular space of the muscle (e.g. from 13 to 10%) would further falsify the estimated intracellular [Na]. Nevertheless, neither the tracer calculations nor the analyses suggest that in 40 min at 1° C the internal [Na] rises by more than about 3 mM, which is under half the increase that can apparently be tolerated at room temperature before the response to Na-free solutions is abolished. In view of the facts that the Na-free effect disappears under conditions when internal [Na] rises, and can be restored by prolonged exposure to Li Ringer's solution, it is hard to avoid concluding that internal [Na] is an important factor in deciding whether or not the effect is seen. However, another temperature-dependent factor may be involved, so that the critical level of internal [Na] also alters with temperature. This suggestion is, of course, supported by the experiment shown in the upper part of Fig. 9, where the Na-free effect finally reappeared at room temperature, although the level of internal [Na] can then have differed little from that during the previous negative test at the low temperature.

Variation in Na influx with external Na concentration

In the absence of evidence to the contrary it has usually been assumed in discussions of the Na fluxes in muscle (see, for example, Harris & Burn, 1949; Keynes, 1954) that the influx is directly proportional to the external [Na]. It seemed of interest to test the correctness of this assumption, and at the same time to see whether the way in which the Na influx varied with external [Na] fitted with predictions based on the curve in Fig. 5. The method adopted was to expose paired sartorii to two solutions containing the same amount of ^{24}Na but different total amounts of Na, one always having the normal 111·2 mM-Na while the other only had from 0·5 to 33% of this, equimolar quantities of Li being substituted for the remaining Na. After exactly 10 min in the tracer solution (during which the muscle was moved up and down once per second to ensure good stirring), each muscle was removed, rinsed very briefly in an inactive medium to remove superficial ^{24}Na, drained, and then transferred in the usual way through a series of samples of normal inactive Ringer's solution; finally the muscle was weighed, disintegrated in HNO_3, and its residual radioactivity was counted. After adding up the counts in reverse order, the total radioactivity in the muscle was plotted against time of soaking in the inactive solution, the counts being corrected for decay of the ^{24}Na so that zero time was the same for both muscles. On some occasions the control ^{24}Na solution with normal [Na] was used first, and on others the first muscle was put into the test solution with low [Na]. The results of an experiment in which the first muscle spent 10 min in a ^{24}Na solution containing 11·1 mM-Na and 100·1 mM-Li (plus the usual K and Ca), and the second 10 min in a ^{24}Na solution with 111·2 mM-Na, are plotted in Fig. 13.

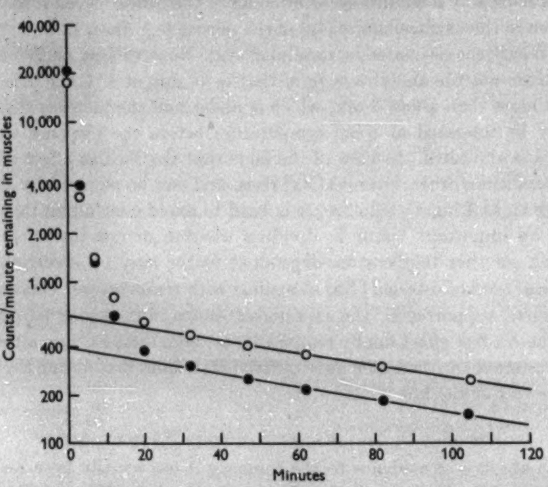

Fig. 13. The effect of external [Na] on Na influx into a pair of sartorius muscles. ●, muscle weighing 58·8 mg had spent 10 min in a solution containing 1 part Na* Ringer's solution + 9 parts normal Na Ringer's solution, temperature 18·8° C. ○, muscle weighing 64·1 mg had spent 10 min in a solution containing 1 part Na* Ringer's solution + 9 parts Li Ringer's solution, temperature 19·0° C. Counts were taken in normal inactive Ringer's solution at 16·9 °C, corrected for decay of ^{24}Na to same zero time. Specific activity of Na* Ringer's solution was 1 count/min equivalent to 6·86 pmoles Na. For further details see Table 4.

It will be observed that both muscles gave diphasic curves for the washing out of radioactivity, with a fast phase whose half-time was 1·3 min (rate constant 32 hr^{-1}) and a slow phase whose half-time was 74 min (rate constant 0·56 hr^{-1}), presumably corresponding to the exchange of extra- and intracellular Na, respectively; but that the muscle which had been in the labelled solution containing only 10% of the normal [Na] had gained 1·5 times as much intracellular ^{24}Na as the control. This difference was not the result of accidental variation between the two muscles, since closely consistent results were obtained in a number of other pairs of muscles (see Table 4), and when, as a check, the members of one pair were both exposed to the control ^{24}Na solution, the two sets of points coincided almost perfectly. It may be concluded that the size of the Na influx is not directly proportional to the external Na concentration, for when [Na] is lowered to 10% of its normal value, Na influx is only reduced to 15% of normal. Another way of looking at the result is to say that it demonstrates that there is normally some degree of competition between Na$^+$ ions attempting to enter the muscle fibres, since it shows that fewer labelled ions penetrate into the fibres when all the external ions are Na$^+$ than when 90% of them are Li$^+$.

TABLE 4. Na influxes into frog sartorius muscles

Influx in normal Ringer's solution (pmole/cm^2.sec)	Rate constant for loss of ^{24}Na (hr^{-1})	Internal [Na] for steady state (m-mole/l. myoplasm)	% normal external [Na] for paired muscle	Relative influx of ^{24}Na	Observed % normal influx	Predicted % normal influx
2·6	0·53	8·9	0·5	2·01	1·0	1·1
2·6	1·00	4·7	1	1·74	1·7	2·2
4·0	0·66	10·9	3	1·75	5·2	6·6
3·5	0·80	7·9	5	2·06	10·3	10·5
1·9	0·56	6·1	10	1·52	15·2	19·3
7·2	1·43	9·1	20	1·22	24·4	35·0
3·2	0·77	7·5	33	1·11	36·6	50·0
9·0	1·48	11·1	100	1·00	100	100
Mean 4·2	0·90	8·3	—	—	—	—

The basis for calculating the figures in the last column is described in the text. Temperature 16–21° C.

In order to calculate the relative sizes of the influxes in the two muscles, and the absolute size of the influx in the control muscle, the results were plotted semi-logarithmically, straight lines were fitted to the points between 30 and 90 min, and the slow phases were then extrapolated to zero time, giving figures for the intracellular counts/min gained during 10 min in the radioactive solution, and hence for the rate of gain of activity in counts/min per second. From the specific activity of the labelled Na (in counts/min per pmole) and the total surface area of the muscle fibres (worked out from the muscle weight, taking the fibre space as 87%, density as 1·05 g/cm^3, and fibre diameter as 80μ, i.e. assuming a surface:weight ratio of 0·415 cm^2/mg), the influxes could then be expressed in pmoles/cm^2.sec. The values thus obtained are shown in Table 4.

An unavoidable disadvantage of working with whole sartorius muscles is the slowness of equilibration between the ions in the extracellular space and those in the external medium. This might have been responsible for the introduction of appreciable errors in using the simple procedure just described, in which it was tacitly assumed that the external specific activity was identical with that of the external medium throughout the 10 min period of uptake of ^{24}Na. But just as the extracellular radioactivity does not rise instantaneously to the assumed value, so it does not fall immediately to zero at the start of the period of washing out, and some further ^{24}Na has the opportunity to enter the fibres before all the labelled ions are washed away. In the process of extrapolation, therefore, counterbalancing errors are introduced. Since it could not be expected that the errors would cancel one another out exactly, it seemed necessary to work out roughly how large they were likely to be. A complete solution of the appropriate diffusion equations (see Harris & Burn, 1949; Keynes, 1954) was not attempted, partly because of the complications involved, and partly because there was enough uncertainty in deciding on correct figures for the constants needed in applying the solution to detract considerably from its usefulness. Instead, the muscle was treated as if it were simply a single intracellular compartment in series with a single extracellular compartment; such a simplified system gives a washing-out curve closely resembling that of a real muscle, and served well enough to establish the order of size of the probable errors. The results of the calculation will not be presented in detail, but its conclusion was that the over-all error under the conditions of our experiments (e.g. 10 min exposure to ^{24}Na, rate constant for extracellular phase 30–50 hr^{-1}, rate constant for intracellular phase 0·5–1·5 hr^{-1}) was within ±10%. This degree of uncertainty does not seem an unreasonable price to pay for the convenience of working with whole muscles rather than isolated fibres.

As Table 4 shows, the average influx in normal Ringer's solution was 4·2 pmole/cm^2.sec; this agrees well with the value of 3·5 pmole/cm^2.sec obtained by Hodgkin & Horowicz (1959) for isolated single muscle fibres. The table also shows the internal Na concentrations which would make the Na efflux equal to the influx, taking the mean fibre diameter as $80\,\mu$. Taking the same figures as before for fibre space and muscle density, the average of 8·3 m-mole/l. myoplasm corresponds to 6·9 m-mole/kg muscle. It is interesting to compare this result with that of another method of arriving at the internal [Na]. In many of our experiments the initial period of soaking in radioactive Ringer's solution was long enough for the exchange with ^{24}Na to be virtually complete, and in such cases the total amount of apparently intracellular ^{24}Na could subsequently be added up. The calculation was not always done, but in one group of eleven consecutive experiments the average content of intracellular Na was again 6·9 m-mole/kg. No doubt the agreement between the two estimates is fortuitously exact, but since any diffusion errors in the influx values would give rise to parallel errors in the calculated internal [Na], it provides some confirmation that such errors were not gross ones.

The experiments described earlier in this paper can be interpreted on the supposition that just over half the sodium efflux in ordinary Ringer's solution involves a one-for-one Na/Na exchange, while the remaining 47·5% of the efflux is not immediately affected by removing external Na. On this hypothesis it is possible to predict, from the curve in Fig. 5 relating Na efflux to external [Na], how the Na influx should vary with external [Na]. The figures in the last column of Table 4 were arrived at by assuming the Na influx to consist of two components: (*a*) an 'independent' component increasing linearly with external [Na], and contributing 47·5% of the total influx when [Na] = 111·2 mM, and (*b*) a 'coupled' component increasing non-linearly in a way determined by the curve in Fig. 5, and contributing 52·5% with 111·2 mM-Na outside the fibres. In the penultimate column of Table 4 are to be found the observed influxes in the low [Na] solutions, each expressed as a percentage of the influx for the paired control muscle. It will be seen that on the whole the observed and calculated percentages agree fairly well with one another. The tendency for the observed percentages to be slightly lower than the predicted ones is partly attributable to the diffusion errors discussed above, since the calculations suggested that the influxes were probably over-estimated to about the same absolute extent in both the control muscles and the muscles in low-Na solutions, so that their ratios were apt to be slightly underestimated.

DISCUSSION

The distribution of sodium in frog muscle

Carey & Conway (1954) have suggested that the Na of frog muscle is made up of three fractions, which they describe as follows: (1) 14 m-mole/kg of interspace Na; (2) 8 m-mole/kg of 'special region' Na; (3) 2 m-mole/kg of 'general fibre' (i.e. intracellular) Na. Their 'special region' is a part of the muscle considered to have a high permeability both to Na and to K and concentrations of Na higher, and of K lower, than inside the majority of the muscle fibres. In order to avoid misunderstanding, it seems advisable that before proceeding with the interpretation of the results some figures should be given for the apparent distribution of muscle Na in our experiments, since there is some conflict between our values and those of Carey & Conway. Analyses of six muscles dissected in normal Ringer's solution (solution *A*, Table 1) gave a total Na content of 25·3 m-mole/kg, which fits well with Boyle & Conway's (1941) value of 23·9 m-mole/kg, since their Ringer's solution contained slightly less Na than ours. Taking the extracellular space as 13%, there would be 14·5 m-mole/kg of interspace Na, leaving 10·8 m-mole/kg to be accounted for. The Na fraction with which this paper is concerned, and which we classify as the 'intracellular' fraction, is that which exchanges with a time constant of the order of 1 hr; as mentioned on p. 616, it amounts to 6·9 m-mole/kg. In all our measurements, radioactivity lost during the first 25 min after removing the muscles from the labelling solution was ignored; this would presumably include any 'special region' Na, exchanging more slowly than extracellular Na, but faster than fibre Na proper. However, it only seems necessary to allocate about 2 m-mole/kg under this heading, since the work of Harris & Steinbach (1956) suggests that of the final 3·9 m-mole/kg nearly 2 m-mole/kg is probably situated in the connective tissue and is so tightly bound that it only exchanges very slowly with ^{24}Na.

While the existence of some 'special region' Na is not denied, it is suggested that it does not account for quite as large a proportion of the muscle Na as was proposed by Carey & Conway (1954). It is difficult to explain the whole of the discrepancy between the two sets of Na distribution figures, but part of it may arise from a difference in the method of labelling the Na. Carey & Conway injected ^{24}Na into living frogs and left them to equilibrate until the next day, whereas we usually labelled the muscles by soaking in radioactive Ringer's solution for 3·5 hr. Their data therefore refer to muscles examined appreciably sooner after killing the frogs. Since, as is shown by Table 3, muscles exposed to Ringer's solution do slowly gain Na, muscles from injected frogs may well contain relatively less Na inside their fibres at the beginning of the period of washing out.

The coupling of the ionic fluxes

The most obvious explanation for a reduction in the sodium efflux when an external ion is removed is that there is some form of coupling between the outward movement of Na^+ ions and the inward movement of the ion in question. An alternative type of mechanism would be that enzymes involved in active transport and situated on the outer surface of the cell require the presence of various activating ions in the external medium in order to fulfil their function. But, at least in cephalopod axons, the existence of a loose linkage between K influx and Na efflux seems the more promising hypothesis (Hodgkin & Keynes, 1954). The results described here thus suggest that in frog muscle the outward movement of labelled Na is coupled both to an inward movement of Na and to an inward movement of K. Neither suggestion is a new one, the Na–Na linkage having been proposed some time ago by Ussing (1949) and the Na–K linkage by Keynes (1954). However, there has not previously been any direct experimental evidence to support Ussing's exchange diffusion idea. Another new finding is the tendency of the Na- and K-coupled components of the Na efflux to vary in a reciprocal fashion, apparently in response to changes in internal [Na]. This probably explains why it was originally easier to demonstrate the effect of K-free solutions on Na-loaded muscles than on freshly dissected ones (Keynes, 1954).

There is still no indication as to the nature, on a molecular level, of the coupling between the fluxes. The results in Fig. 6 show that complete independence of the Na-linked and K-linked effluxes of Na should not be assumed and the whole of the Na efflux cannot, in any case, be tightly coupled to Na or K entry, since there is still a substantial efflux of Na into a solution containing neither Na nor K. It may be worth while to point out that the kind of circulating carrier mechanism for coupled active transport of cations that has been discussed by several authors (see, for example, Glynn, 1957) could, if there were a passage back of the lipoid-soluble carriers either combined with Na^+ or uncombined with a cation, give rise to the appearance of Na-coupled or uncoupled outward fluxes of Na.

The two-compartment hypothesis

It seems to us that the marked non-linearity of the curve relating Na efflux in Li Ringer's solution to internal [Na] should provide a useful clue concerning the mechanism of the Na pump, and that it is therefore worth while to inquire into the possible reasons for this behaviour. The first hypothesis to be considered is that which was used in fitting the curves in Fig. 4 *B* and *C*—namely, that the internal Na behaves as if it were confined in two distinct compartments, the efflux from one of these being unaltered in Li Ringer's solution while the efflux from the other is highly sensitive to removal of external Na. The two main points in favour of this idea are that it fits the experimental curves well (though this is not a strong argument, since there are several arbitrary constants to be chosen), and that it more or less correctly predicts the large rise in efflux on restoration of external Na. The two main points against it are (1) the necessity to suppose that in normal Ringer's solution the two compartments fortuitously have equal rate constants for exchange of labelled Na, and (2) the behaviour of the Na efflux on finally testing a second time in Li Ringer's solution after a preliminary long exposure to Li followed by a period in normal Ringer's solution. Possibly (1) does not raise an insuperable difficulty, because it is well known (see, for example, Harris, 1950) that in many muscles the slow phase of Na exchange in normal Ringer's solution appears to be the sum of at least two exponentials, and those in which the slow phase is well fitted by a single exponential (see Keynes, 1954, Fig. 4) may be the exception rather than the rule. The second objection is harder to surmount. On the suggested form of the hypothesis, in which the two Na fractions are free to exchange with extracellular Na *in parallel* with one another, the substantial rise in efflux on restoring external Na after a long exposure to Li occurs because by then the Li-insensitive fraction is almost wholly depleted of its radioactivity. Since this fraction cannot regain any ^{24}Na during an ensuing exposure to normal external [Na], one would expect a final exposure of the muscle to Na-free Ringer's solution to produce a drop in efflux just as big as the immediately preceding rise, reflecting chiefly the high sensitivity to Li of the other compartment. Yet experiments like that illustrated in Fig. 2 suggested that the final response was roughly the same as the initial one, instead of being appreciably larger. This observation seems to make the two-compartment hypothesis unacceptable in its present form, despite its other merits.

A rather different sort of two-fraction system would be one in which each fibre could be regarded as a single compartment containing Na in two different forms—say (1) freely ionized, and (2) bound to an organic molecule by a covalent linkage. This type of system has often been considered speculatively in attempts to account for departures from single-compartment behaviour.

There is undoubtedly some 'bound' sodium in muscle, as has been demonstrated by Harris & Steinbach (1956); but they were mainly concerned with Na apparently in the connective tissue, held so firmly that under the conditions of our experiments it would never have exchanged to any appreciable extent with the ^{24}Na in the first place. In any case, while the presence of a sodium-complexing agent inside the fibres could lead to the relationship shown in Fig. 4*B* and *C* between Na efflux and total internal [Na], it would seem inadequate to explain the final large rise in efflux seen in Fig. 4*A*, since there is no obvious way in which a change in [Na] *outside* the membrane could make itself felt on the behaviour of combined Na *inside* the cell.

A single-compartment hypothesis

Since the results of the Li experiments cannot be explained satisfactorily on a two-compartment basis, it is next necessary to see whether there is any single compartment arrangement that might do better. It is possible, in fact to envisage a physically plausible mechanism for extruding sodium from a single compartment which would explain some, though by no means all, of our findings. Thus it may be supposed that there are carrier molecules in the membrane which in Na-free solutions can only move from inside to outside in combination not with 1 but with n Na^+ ions, some of which are discharged at the outer surface. Their movements will give rise to an efflux of Na whose size will be proportional to the nth power of the internal [Na], and on plotting log. efflux against log. [Na] a line with slope n should be obtained. In Fig. 14 the results of four experiments (one from Fig. 2, two from Fig. 4, and one other) have been plotted in this fashion, and it will be seen that three of them can be fitted approximately by straight lines whose slope is close to 3, while the fourth has a similar slope over most of its length, but deviates significantly at the lower end. It follows that the behaviour of the efflux of Na which is observed to continue in Li Ringer's solution might be explained if it involved the transfer of Na^+ ions through the membrane in groups of three. Assuming that the 'cube law' efflux is unaffected by removal of external Na, it has then to be supposed that in normal Ringer's solution labelled Na can also emerge from the fibres through the operation of an exchange diffusion mechanism, which is wholly or partly blocked in Na-free solutions. In order to account quantitatively for the large rise in efflux on restoring external Na after a long exposure to Li, the size of the exchange diffusion component needs to be taken as directly proportional to the internal [Na]. Thus in one of the experiments of Fig. 4 the reduction in efflux on first transferring the muscle to Li Ringer's solution was of the order of 370 counts/min^2, and at this time the total radioactivity in the muscle was about 37,000 counts/min. The rise in efflux on going back to normal Ringer's solution later on was roughly 130 counts/min^2, the total radioactivity then being 11,000 counts/min. The component of the efflux which was abolished by removing external Na had therefore decreased to 0·35 of its initial value while the internal radioactivity fell to 0·30. In the other experiment of Fig. 4 the corresponding changes were to 0·45 and 0·58 of the initial values. Hence in both experiments a direct proportionality between the amount of efflux cut off in Li Ringer's solution and the total internal radioactivity would fit the figures fairly well.

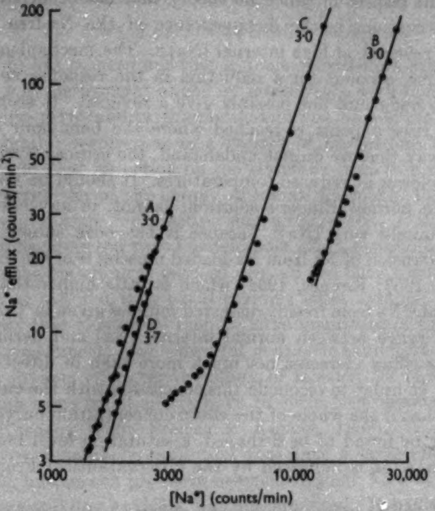

Fig. 14. Log-log. plot of the ^{24}Na efflux into Li Ringer's solution against the amount of ^{24}Na in the muscles. *A*, values from Fig. 4*B*. *B*, values from Fig. 4*C*. *C*, values from an experiment not described elsewhere, in which the initial internal [Na] was made rather higher than usual by equilibrating the muscle in K-free ^{24}Na Ringer's solution. *D*, values from Fig. 2. The number against each set of points is the slope of the straight line.

It would obviously be dangerous to lay much stress on the agreement between a cube law and the decline of Na efflux as a function of total internal [Na]. The range of internal [Na]'s covered was not very large, and the fit with straight lines in Fig. 15 was by no means perfect, particularly in one of the experiments. There are doubtless other mechanisms which would give similar efflux–concentration curves, though we are unable to think of any as simple as the one we have chosen. But any single-compartment hypothesis seems to have the advantage of accounting satisfactorily for the observation which was the main stumbling-block to the two-compartment hypothesis, that is to say for the tendency of the Na-free response to revert towards normal at the end of experiments like that illustrated in Fig. 2. This is so because when the muscle is returned to normal Ringer's solution with reduced internal [Na] after a long period in Li Ringer's solution, the Na influx will be much larger than the Na efflux, at least to start with, and the internal [Na] will therefore rise quickly again towards its steady-state level. The extent of the change in final Li response after varying intervals in normal Ringer's solution has not been examined in detail, but appears to fit roughly with this explanation.

An important respect in which no theory that has occurred to us will explain our observations is the disappearance of the Na-free response, and sometimes its reversal, at high internal [Na]'s. The mechanism just outlined would, of course, account for a reduction in the response as internal [Na] was increased, but could not possibly give a reversal. It seems that as the internal [Na] rises a point is reached where the behaviour of the system changes in a way that we do not understand, the critical level at which this happens being lower at reduced temperatures. It should be realized that the efflux of Na in normal Ringer's solution, cannot, in any case, continue to increase indefinitely with $[\mathrm{Na}]^3$, because it has been shown that the rate constant for extrusion of Na from Na-loaded muscles is of the order of 1·2 hr^{-1} (see Desmedt, 1953; Keynes, 1954) which is little higher than the average figure for loss of ^{24}Na from freshly dissected muscles given in Table 4, implying that over the range between normal internal [Na] and perhaps five times normal the Na efflux increases not much more than in direct proportion to internal [Na]. In order to reconcile this conclusion with the cube law, it may be suggested that if the whole of the efflux-concentration curve were investigated it might be found to be S-shaped, a saturation level being reached at the higher [Na]'s, as is indicated by the observations of Frazier & Keynes (1959).

Comparison with other tissues

Other evidence in support of Ussing's (1949) exchange diffusion hypothesis as applied to Na movements is rather scanty. Hodgkin & Keynes (1955) concluded that in *Sepia* axons there did not seem to be any appreciable exchange diffusion of Na, since the efflux of ^{24}Na was increased by 20–50% in Na-free solutions rather than being cut down. They did not use Li as a substitute for Na, but it has since been confirmed in squid axons that Li and choline have very similar actions on the Na efflux; in one axon the efflux of ^{22}Na was increased by 38% in Li sea water and by 45% in choline sea water (P. C. Caldwell & R. D. Keynes, unpublished experiment). The normal internal [Na] in these cephalopod axons is probably about five times higher than in freshly dissected frog muscle, and it is possible that if it could be reduced to a much lower level their behaviour might become more like that described here for frog muscle. In human erythrocytes, another situation where internal [Na] is relatively low, Glynn (1956) found evidence that the efflux of ^{24}Na into a K-free medium was considerably reduced by substituting choline for most of the external Na. Croghan (1958) also reported the occurrence of Na exchange diffusion, in this case between an intact organism, the brine shrimp *Artemia salina*, and its surroundings. When erythritol was substituted for external Na, the efflux of ^{24}Na from the animals decreased sharply and reversibly, but there were indications that K could replace Na to some extent, and the mechanism involved is not necessarily the same as that in the cell membrane.

The effect of lithium on cellular metabolism

Shanes (1956) has pointed out that in ionic substitution experiments some of the effects may be brought about by interference with metabolism rather than by specific permeability interactions in the membrane. Since Li has been said to inhibit respiration in a variety of tissues (see Schou, 1957) it might be thought that its action on the Na efflux from frog muscle could be due to an interruption of the supply of metabolic energy to the Na pump. There are several reasons for rejecting such a suggestion. If it were correct, it would have to be supposed that Li could only affect metabolism from outside the cell, since the Na efflux is reduced virtually at once when external Na is removed, and is restored with equal rapidity when external Na is replaced, despite the fact that there may then be large quantities of Li inside the cell. Moreover, in frog muscle even powerful metabolic inhibitors like CN do not have a very obvious effect on the efflux of labelled Na (Keynes & Maisel, 1954), although some small effects have recently been demonstrated (Carey, Conway & Kernan, 1959; Frazier & Keynes, 1959). It is true that in cephalopod axons metabolic inhibition does have a large effect (Hodgkin & Keynes, 1955), but, as has just been mentioned, their Na efflux is apparently stimulated by removing external Na, not inhibited. It has also been shown (P. C. Caldwell & R. D. Keynes, unpublished) that even when the [Li] inside a squid axon is suddenly raised to more than 40 mM by micro-injection, there is no discernible change in the efflux of ^{22}Na.

The inhibitory effect of Li on the efflux of Na from frog muscle could arise either because Li has a much lower affinity than Na for some component of the transport mechanism, and is therefore unable to exchange with Na, or because it has a much higher affinity than Na and succeeds in displacing it from the membrane. The observation just quoted clearly supports the first possibility, as does the ability of the Na efflux in frog muscle to rise markedly on restoration of external Na at a time when there is still a high internal [Li]. The fact that Na substitutes as different in their chemical natures as Li and choline have similar actions both in frog muscle and in squid axons, also fits better with the idea that they are simply ignored by the active transport mechanism than that they combine too well with it. The Na carriers seem unlikely to discriminate against Li and choline to exactly the same extent, and there was some indication (see p. 595) that the efflux of Na was reduced slightly less by choline than by Li. The following paper (Keynes & Swan, 1959) suggests that selection against Li is not 100% effective, since there is apparently a slow passive Na-Li exchange in muscles soaked in K-free solutions; furthermore, it seems that Li can be actively extruded from the muscle fibres, although at a rate 10–25 times slower than Na. Perhaps the inhibitory action of Li on metabolism is a result of permeability differences rather than a cause, for if the rate of respiration of muscle depends to any great extent on the rate of consumption of energy by the Na pump, then a long exposure to Li may reduce respiration by gradually slowing down the rate at which cations are transported out of the fibres.

SUMMARY

1. When Na in the external medium is replaced by choline or Li, the efflux of ^{24}Na from freshly dissected frog muscles is reversibly reduced to about half. It is concluded that in these muscles roughly half the efflux of labelled Na involves a coupled Na/Na exchange of the type proposed by Ussing (1949).

2. In muscles stored in normal Ringer's solution for more than 12 hr, or in K-free Ringer's solution for an hour or two, the response to Na-free solutions disappears. At the same time the reduction in ^{24}Na efflux caused by removal of external K becomes relatively greater. Both changes can be reversed by leaving the muscles for some time in Li Ringer's solution, and are apparently related to a rise in the Na content of the muscle fibres.

3. The Na-free effect also disappears on cooling to 10° C or below, but again can be restored by long exposure to Li Ringer's solution. It is suggested that the effect of cooling arises partly from a tendency for internal [Na] to rise at low temperatures, and partly from a lowering of the critical [Na] at which the Na-free response vanishes.

4. It is shown that the fraction of the Na efflux dependent on the presence of Na in the external medium varies with external [Na] in Michaelis-Menten fashion, with half-saturation at about 40 mM.

5. The influx of Na into sartorius muscles is not linearly related to the external [Na], being about twice as great at low [Na]'s as would be expected for a direct proportionality. The way in which the influx varies fits roughly with the idea that in normal Ringer's solution half the labelled Na$^+$ ions moving inwards are doing so through the operation of an exchange diffusion mechanism.

6. The efflux of Na from muscles soaked for long periods in Li Ringer's solution is not linearly related to internal [Na], the curve obtained being approximately fitted by a cube law. At the end of a long period in Li Ringer's solution the Na efflux may increase as much as tenfold on restoring external Na.

7. An interpretation of the results in terms of a system consisting of two compartments in parallel is discussed and rejected.

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THE PERMEABILITY OF FROG MUSCLE FIBRES TO LITHIUM IONS

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When Overton (1902) studied the excitability of frog muscle in different media, he found that lithium was the only cation which could be substituted for sodium without causing the fibres to become inexcitable. This suggests that in some ways the behaviour of the muscle membrane towards lithium must be similar to its behaviour towards sodium. In other respects, however, the membrane discriminates between sodium and lithium, as is shown by the experiments described in the preceding paper (Keynes & Swan, 1959) on the effect of lithium on the efflux of labelled sodium from frog muscle. The results of this work on the sodium efflux could not be interpreted without knowing more about the rate of movement of lithium ions through the membrane, and this paper is therefore concerned with some measurements of the lithium influx into freshly dissected muscles, and with the rate at which lithium moves outwards from lithium-loaded muscles. Since lithium has no usable radioactive isotopes (the half-life of ^{8}Li is under 1 sec), we were only able to measure the net rate of transfer of lithium into and out of the muscles, using a flame photometer to determine the lithium contents of muscles treated in various ways. But some useful figures were obtained for the resting influx of lithium from a solution similar in its composition, except for the substitution of Li for Na, to normal frog Ringer's solution, and the extent to which lithium can be actively extruded from the interior of the muscle fibre was also studied. Finally, a few observations were made with intracellular micro-electrodes to see whether there was any obvious difference between the action potentials recorded in Na and in Li Ringer's solution.

METHODS

Material

Pairs of sartorius muscles were dissected from small specimens of *Rana temporaria*, and tied to glass racks at their body length, as described by Keynes & Swan (1959). In order to minimize interfibre diffusion times, muscles weighing between 20 and 40 mg were preferred, but a few larger muscles were used on occasions when no small frogs were available. The muscles were dissected as cleanly as possible at the pelvic ends, leaving only enough connective tissue for them to be tied to the racks without crushing the ends of the fibres. When there was any suspicion that either muscle of the pair had been damaged in the course of dissection, both were rejected. They were also rejected if at the conclusion of the experiment either muscle contained more than about half a dozen fibres whose myoplasm was visibly clotted. Excitability was checked visually at the beginning and end of each experiment; except in cases where the final solution to which they were exposed contained 10 mM-K, they always gave good twitches, even when the experimental soaking procedure had lasted for nearly 3 days. The experiments were done during the months of March, July and August.

Solutions

For the determinations of lithium influx, and for recording action potentials, the 'normal' Ringer's solution contained 2·5 mM-K and had the composition shown in Table 1. In order to load the muscles with Na or Li, they were sometimes soaked at a low temperature in the low-K 'soaking-in' solution shown in Table 1, and sometimes for a shorter period in a similar solution from which the potassium phosphate was omitted. The 'sodium recovery' solution was similar to the medium devised by Boyle & Conway (1941) to imitate closely the inorganic composition of frog plasma, except that (following Desmedt, 1953), it contained 10 mM-K. On some occasions a K-free modification of it was used, the KCl being omitted, but the other components left unchanged. The 'lithium recovery' solution had Li substituted for all the Na except the 0·9 mM added as gluconate; since $LiHCO_3$ was not available as such, it was made up by taking appropriate quantities of 'Specpure' Li_2CO_3 and HCl A.R. Both recovery solutions were equilibrated with 3 or 5% CO_2 in O_2.

TABLE 1. Composition of solutions (mM)

Normal Ringer's		Soaking-in solution		Sodium recovery medium		Lithium recovery medium	
NaCl or LiCl	111·2	NaCl or LiCl	120	NaCl	73	LiCl	78·5
K_2HPO_4	1·14	K_2HPO_4	0·125	Na_2HPO_4	2·5	K_2HPO_4	2·5
KH_2PO_4	0·23	KH_2PO_4	0·025	NaH_2PO_4	0·5	KH_2PO_4	0·5
$CaCl_2$	1·8	$CaCl_2$	0·9	KCl	10	KCl	4·5
				$CaCl_2$	0·9	$CaCl_2$	0·9
				$NaHCO_3$	25	$LiHCO_3$	25
				$MgSO_4$	1·5	$MgSO_4$	1·5
				Na gluconate	0·9	Na gluconate	0·9
				Glucose	26	Glucose	26

Analyses

After the muscles had been exposed to the various solutions as described under 'Results', they were cut off the glass racks and their excitability was checked. They were next thoroughly blotted, a few millimetres at either end was cut off to minimize errors from connective tissue and remnants of attachments to other muscles, and their central parts were put into platinum crucibles, in which they were weighed. After drying at 110° C, they were reweighed, and were then dry-ashed during the night in an oven at about 600 °C. The ash was dissolved in a drop of strong HCl (the ash of Li-treated muscles did not dissolve completely in pure water, perhaps because of the relative insolubility of Li_2CO_3), and the crucibles were then nearly filled with distilled water; their capacity was slightly over 5 ml. The exact dilution for each muscle was determined by weighing the crucibles, and 1·0 ml. samples were next taken by pipette and made up to 25 ml. in standard flasks for Na and K determinations with an EEL flame photometer. The sensitivity of the flame photometer for Li was appreciably less than for Na, so that to determine Li it was necessary to spray fluid directly from the crucibles, the 4 ml. remaining being quite enough to take readings in triplicate.

RESULTS

The lithium influx into frog muscle

The main problem in measuring the influx of lithium into sartorius muscles was that of discriminating between lithium which had merely exchanged with the sodium in the extracellular space of the tissue, and that which had entered into the interior of the fibres. Two methods of approach were tried, giving results that were in tolerable agreement. The first consisted in analysing pairs of muscles after one had been exposed to Li Ringer's solution for about 0·5 hr, which should have been long enough for all the extracellular Na but not much of the intracellular Na to have been replaced by Li (see Na values in Table 2), and after the other had been in the same solution for a further 2 hr. The difference between their Li contents was taken to represent Li that had penetrated into the fibres during the extra 2 hr. The disadvantage of this technique was that it depended on there being a good correlation between the amounts of extracellular Li in the members of each pair. However, the figures in Table 2 show that despite appreciable variation in total Li content between the pairs, in six cases out of seven the muscle exposed for the longer period contained slightly more Li than its companion. The average rate of gain of Li in the seven experiments was 0·029 m-mole/kg.min. Taking the muscle density as 1·05 g/ml., the extracellular space as 13 %, and the mean fibre diameter as 80μ (see Keynes, 1954), 1 kg of frog sartorius contains 830 ml. of fibres whose total surface area is $4{\cdot}15 \times 10^5$ cm^2. The average lithium influx was therefore about 1·2 pmole/cm^2.sec.

TABLE 2. The rate of gain of lithium in pairs of muscles soaked for various periods in a solution containing 111·2 mM-Li and 2·5 mM-K

Muscle *A*			Muscle *B*			
Time in Li (min)	Li content (m-mole/kg)	Na content (m-mole/kg)	Time in Li (min)	Li content (m-mole/kg)	Na content (m-mole/kg)	Rate of gain of Li (m-mole/kg.min)
24	25·6	—	158	24·6	—	−0·007
24	16·7	2·9	144	22·6	1·3	+0·049
24	19·1	2·7	144	24·5	2·2	+0·045
24	31·0	4·1	144	39·0	3·4	+0·067
30	15·8	9·2	150	17·0	5·3	+0·010
30	17·5	5·7	150	20·3	2·8	+0·023
30	17·9	7·9	150	19·7	3·3	+0·015
Mean	20·5	5·4	—	24·0	3·1	+0·029

The second method involved the exposure of muscles to Li Ringer's solution for about 2 hr, followed by a period of washing in normal Ringer's solution long enough to ensure removal of all the extracellular Li, and finally analysis of the Li remaining in the muscles. The experiments on Li efflux described in the following section showed that once Li had entered the fibres it was not lost very rapidly, so that it could be expected that 30 min of washing in normal Ringer's solution would not cause a serious depletion of the intracellular Li. As a check on this assumption the muscles were treated in pairs; both were soaked in Li Ringer's solution for the same period, and one was then washed in normal Ringer's solution for 30 min, the other for about 70 min. It may be seen from Table 3 that during the extra 40 min of washing the amount of lithium in the muscles only decreased by about 15%. The Li contents of the muscles in group *A* should therefore represent a fairly reliable estimate of the intracellular Li gained during 2 hr in Li Ringer's solution (if the muscles had still contained traces of extracellular Li, this would offset any slight losses of intracellular Li during the 30 min period of washing). The average rate of gain of Li for 5 muscles was 0·055 m-mole/kg.min. On the same basis as before, this corresponds to an influx of 2·2 pmole/cm^2.sec. Although the second method therefore gives a slightly higher average figure than the first, the individual values overlap one another (see Tables 2 and 3) and there is no essential disagreement between the two techniques.

TABLE 3. Lithium content of pairs of muscles first soaked about 2 hr in a solution containing 111·2 mM-Li and then washed for various periods in normal Ringer's solution

	Muscle *A*		Muscle *B*		
Time in Li (min)	Washing time (min)	Li content (m-mole/kg)	Washing time (min)	Li content (m-mole/kg)	Rate of gain of Li of muscle *A* (m-mole/kg.min)
151	28	7·6	65	6·5	0·050
119	25	8·6	65	7·2	0·072
120	32	7·1	68	5·8	0·059
120	36	5·5	72	4·1	0·046
120	41	5·9	75	6·1	0·049
Mean					
126	32	6·9	69	5·9	0·055

The efflux of lithium from frog muscle

In order to study the rate at which lithium would move outwards from frog muscle fibres, it was necessary to subject the muscles to a preparatory period of loading with lithium. This was done by soaking the muscles at a low temperature (2° C) either for 44–69 hr in a low-K lithium solution (see Table 1), or for 27–40 hr in a similar solution with potassium omitted altogether. The muscles were mounted on racks at their body length, and oscillated up and down in a large volume of solution once per second throughout the period of soaking-in; the solution was changed every 12 hr. At the end of the soaking-in period almost all the sodium in the muscles (see Table 6), and a large part of their potassium (see Tables 4–6) had been replaced by lithium. It is of interest to note in passing that in spite of virtually complete removal of both external and internal sodium (the 1 m-mole Na/kg remaining in the muscles may have been located in the connective tissue, as the inexchangeable fraction described by Harris & Steinbach (1956)), the muscles were still capable of twitching in what appeared from visual inspection to be a normal fashion.

The principle on which all the experiments were based was to determine the starting levels of lithium, potassium and sodium by analysing one muscle from each pair on completion of the preparatory period, and then to determine the changes in cation levels after the second muscle had been exposed for a few hours to a recovery solution. The closeness of correlation between the starting levels in the individual muscles of each pair was not examined specifically, but the remarkably small differences in lithium content for the shortest recovery periods (see Tables 4–6), despite a much greater range of variation between different pairs, suggests that the agreement between paired muscles must have been rather good. In the experiments of Tables 4 and 5, in which the test muscles were treated with recovery solutions containing sodium, the preparatory period was concluded by soaking both muscles for 1 hr at 2° C in a low-K 120 mM-NaCl solution, in order to replace extracellular lithium, and so to make the two members of the pair directly comparable as far as intracellular lithium was concerned. In the experiments of Table 6 this was unnecessary, both control and test muscles being taken directly out of lithium solutions.

When Li-loaded muscles were soaked in a sodium recovery solution containing 10 mM-K (for composition see Table 1), the lithium was found to emerge slowly from the fibres. The analytical values for all three alkali metal cations are given in Table 4, and the changes in lithium with time are also plotted in Fig. 1. In order to make some allowance in Fig. 1 for variation in starting levels, the lithium contents of the test muscles are expressed as percentages of the contents of the control muscles. The average lithium content of the nine controls was 50·4 m-mole/kg, and the average initial rate of loss of lithium was of the order of 4 m-mole/kg.hr. The results are not sufficiently precise to reveal the exact time course of lithium loss, but an exponential with a time constant of just over 10 hr fits the data fairly well (a straight line would give an equally good fit, but it seems more likely that the disappearance of lithium pursues a roughly exponential course). The average rate constant for lithium loss was 0·079 hr^{-1}; ignoring the two muscles for which the recovery periods were shortest and the values therefore least reliable, the average was 0·095 hr^{-1}. This may be compared with the much faster loss of sodium from Na-loaded muscles allowed to recover in an identical solution. Desmedt's (1953) results correspond to an initial rate constant for sodium loss of about 1·2 hr^{-1} (see Keynes, 1954). The outward movement of lithium was thus over ten times slower than the outward movement of sodium under similar conditions.

To check that the muscles used in our experiments were capable of a rapid net sodium extrusion, six pairs of muscles were loaded with sodium by soaking in low-K 120 mM-NaCl, and one muscle of each pair was allowed to recover for 1–3 hr in the high-K recovery medium. The average starting levels were 64·4 m-mole Na/kg and 48·8 m-mole K/kg; after recovery the test muscles all contained substantially less sodium and more potassium, the final levels being 36·1 m-mole Na/kg (a loss of 28·3 m-mole/kg) and 81·3 m-mole K/kg (a gain of 32·5 m-mole/kg). These figures agree satisfactorily with

TABLE 4. Changes in cation content (expressed in m-mole/kg wet weight) of pairs of Li-loaded muscles soaked for various periods at room temperature (17–22° C) in a high-K sodium recovery medium

Recovery time (hr)	Dry weight (%)	Contents of			Change in			Rate constant for loss of Li (hr^{-1})
		Li	K	Na	Li	K	Na	
0	16·8	74·1	11·5	27·4				
0·8	16·1	75·1	17·1	26·9	+ 1·0	+ 5·6	−0·5	—
0	20·0	37·3	35·4	37·5				
1·3	21·2	35·1	48·9	27·6	− 2·2	+13·5	−9·9	0·047
0	18·5	59·0	24·0	32·0				
2·1	19·4	49·2	42·8	27·4	− 9·8	+18·8	−4·6	0·087
0	22·6	40·0	59·1	16·5				
2·5	21·8	29·2	80·2	17·7	−10·8	+21·1	+1·2	0·126
0	20·9	45·9	36·4	31·1				
3·1	19·8	36·4	56·0	31·9	− 9·5	+19·6	+0·8	0·075
0	21·4	43·3	51·0	18·4				
4·0	21·7	28·5	84·5	17·9	−14·8	+33·5	−0·5	0·105
0	19·0	49·3	35·2	33·5				
4·4	19·8	30·5	60·9	28·2	−18·8	+25·7	−5·3	0·092
0	19·1	41·5	52·0	23·8				
5·5	19·4	25·9	84·0	20·4	−15·6	+32·0	−3·4	0·086
0	18·5	63·2	19·3	29·7				
6·3	18·7	35·1	58·1	31·0	−28·1	+38·8	+1·3	0·093

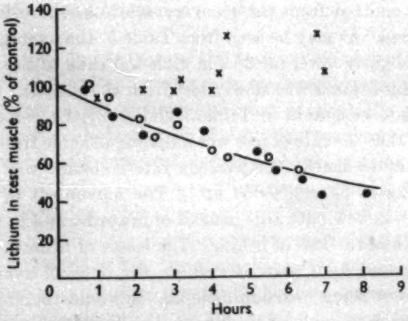

Fig. 1. The decline with time of the Li contents of test muscles. ○, in a high-K sodium recovery medium. ●, in a K-free sodium recovery medium. ×, in a high-K lithium recovery medium. For further details see Tables 4, 5 and 7. Temperature 16–22° C.

Desmedt's observations, but the recovery periods were not short enough to yield further values for the initial rate of sodium loss.

While lithium was lost from the muscles, potassium was regained. Table 4 shows that the movements were not exactly reciprocal, the potassium entry exceeding lithium loss by an average of 11·1 m-mole/kg (S.E. ± 1·3 m-mole/kg). The changes in sodium content, on the other hand, were small and their average (a loss of 2·3 m-mole/kg, S.E. ± 1·2 m-mole/kg) did not differ significantly from zero. The percentage dry weight of the muscles also remained unaltered (average change +0·1 %, S.E. ± 0·3 %), showing that there were no gross volume changes during recovery. The extra gain of potassium was of the right order to have occurred passively in consequence of the type of Donnan equilibrium discussed by Boyle & Conway (1941), the recovery solution being hypertonic relative to the soaking-in solution by just about the 10 mM-KCl that it contained.

Two explanations might be advanced for the observed interchange of lithium and potassium. One would be that the sodium pump is able to extrude lithium actively in exchange for potassium, albeit at under a tenth of the rate at which it can extrude sodium. However, it may well not be legitimate to classify an outward transfer of lithium into a solution containing no lithium as an active transport process, and an alternative hypothesis would be that lithium is somehow exchanged passively for sodium, and that the sodium which thus enters the cell is then extruded in the normal manner in exchange for potassium. These possibilities were examined by doing two further sets of recovery experiments, to see whether lithium could be extruded (*a*) into a K-free sodium recovery solution, and (*b*) into a high-K recovery solution containing lithium instead of sodium.

For (*a*) the procedure adopted was exactly the same as before, but the 10 mM-KCl was omitted from the recovery solution, the other components being left unaltered. As may be seen from Table 5, the average starting level for lithium was slightly lower (at 38·0 m-mole/kg) than in the experiments of Table 4, but again lithium was slowly lost from the muscle. It is clear from the column of rate constants in Table 5 and from the percentage changes plotted in Fig. 1 that the rate of loss was indistinguishable from that observed in the presence of 10 mM-K; the average rate constant was 0·092 hr^{-1} (or, without the first two figures, 0·096 hr^{-1}). The movement of potassium, on the other hand, was now outwards instead of inwards, and in most cases was slightly greater than the loss of lithium. The losses of lithium and potassium were nearly balanced by a gain of sodium, the over-all average change in total cation content being −6·5 m-mole/kg. It would therefore appear that the process by which the bulk of the lithium leaves the fibres is not dependent on a simultaneous inward movement of potassium. Moreover, since the lithium efflux is not blocked by removal of external potassium, as the sodium pump is, lithium seems unlikely to be transported outwards through the channel normally responsible for the net sodium efflux.

It was necessary to verify the correctness of the statement just made that net sodium efflux is blocked in a K-free medium, since this point has apparently not been tested previously (as was pointed out by Hodgkin, 1957). Six pairs of muscles were loaded with sodium by soaking in K-free 120 mM-NaCl at 3° C for 40–44 hr, and one muscle from each pair was then transferred to the K-free sodium recovery solution at 20° C. It will be seen from the analyses listed in Table 6 that in every case the test muscle gained further sodium instead of losing any, and lost a very nearly equivalent quantity of potassium. This established that a net sodium extrusion cannot take place from frog muscle when there is no potassium in the external medium.

TABLE 5. Changes in cation content (in m-mole/kg) in pairs of Li-loaded muscles soaked for various periods in a K-free sodium recovery solution (18–21° C)

Recovery time (hr)	Dry weight (%)	Contents of			Change in			Rate constant for loss of Li (hr^{-1})
		Li	K	Na	Li	K	Na	
0	19·3	29·3	64·5	24·4				
0·7	20·5	28·8	61·0	29·3	− 0·5	− 3·5	+ 4·9	0·026
0	22·6	37·5	73·5	18·2				
1·4	22·1	31·6	65·8	19·3	− 5·9	− 7·7	+ 1·1	0·122
0	20·4	28·2	71·2	25·3				
2·2	19·2	20·8	60·2	35·0	− 7·4	−11·0	+ 9·7	0·139
0	20·5	50·2	46·1	27·0				
3·1	20·6	43·1	34·0	40·5	− 7·1	−12·1	+12·5	0·049
0	21·7	34·9	64·6	26·3				
3·8	21·8	26·6	50·1	34·5	− 8·3	−14·5	+ 8·2	0·072
0	16·8	54·7	34·7	36·6				
5·2	17·4	35·6	18·3	62·0	−19·1	−16·4	+25·4	0·083
0	18·2	33·7	67·0	18·3				
5·7	16·9	18·6	46·7	56·2	−15·1	−20·3	+37·9	0·104
0	19·1	30·7	68·3	21·0				
6·4	18·8	15·3	50·1	51·5	−15·4	−18·2	+30·5	0·092
0	19·1	47·4	31·6	35·2				
6·9	19·0	19·8	13·9	75·1	−27·6	−17·7	+39·9	0·126
0	21·1	33·2	74·0	20·5				
8·1	20·6	14·0	46·4	59·5	−19·2	−27·6	+39·0	0·107

The last set of experiments was designed to see whether lithium-loaded muscles were able to bring about a net extrusion of lithium into a high-K recovery solution in which lithium was substituted for all the sodium except the 0·9 mM added as gluconate (for composition see Table 1). The results set out in Table 7 and plotted in Fig. 1 suggest that in the recovery solution there was either no change in lithium (of the slight loss observed in four of the muscles, about 2·2 m-mole/kg can be attributed to the rather low external lithium concentration in the recovery solution) or a definite rise. There is thus no doubt that frog muscles are unable to extrude lithium at a rate comparable to that at which they can extrude sodium. However, these experiments do not establish unequivocally that there was no movement of lithium whatever against the concentration gradient, since if lithium had been entering the fibres during the recovery period at the same rate as in the influx measurements (order of 3 m-mole/kg. hr), and if the efflux had been zero, some of the muscles should have shown a larger gain of lithium than they did. In other words some of them seem to have been just able to hold their own for several hours in the recovery solution, and this indicates that they were, in fact, able

TABLE 6. Changes in sodium and potassium content of pairs of Na-loaded muscles soaked for various periods at 20° C in a K-free sodium recovery medium

Recovery time (hr)	Dry weight (%)	Contents of K (m-mole/kg)	Contents of Na (m-mole/kg)	Change in K (m-mole/kg)	Change in Na (m-mole/kg)
0	22·6	58·0	55·5		
1·2	22·7	52·0	60·4	− 6·0	+ 4·9
0	19·3	51·5	62·6		
1·8	20·2	48·6	67·7	− 2·9	+ 5·1
0	20·1	52·0	59·0		
2·6	20·0	46·5	64·1	− 5·5	+ 5·1
0	20·9	49·6	61·9		
3·7	20·0	40·3	71·7	− 9·3	+ 9·8
0	21·2	50·0	68·4		
4·8	18·8	27·7	82·4	−22·3	+14·0
0	20·9	68·0	49·8		
6·8	21·6	52·9	63·8	−15·1	+14·0

TABLE 7. Changes in cation content (m-mole/kg) of pairs of Li-loaded muscles soaked for various periods at 16–19° C in a high-K lithium recovery medium. The pairs marked with asterisks had spent 26 hr in a K-free Li solution at 2° C; the others spent 44–51 hr in the low-K Li solution (see Table 1) at the same temperature

Recovery time (hr)	Dry weight (%)	Contents of Li	Contents of K	Contents of Na	Change in Li	Change in K	Change in Na
0	21·0	64·1	55·6	1·0			
1·0	21·3	60·3	51·5	1·2	− 3·8	− 4·1	+0·2
*0	17·8	70·3	60·2	1·5			
2·2	18·5	69·0	60·3	1·7	− 1·3	+ 0·1	+0·2
0	17·9	89·7	27·0	0·9			
3·0	17·9	86·8	33·9	1·1	− 2·9	+ 6·9	+0·2
*0	19·6	57·5	68·0	2·9			
3·2	19·9	59·2	61·0	1·9	+ 1·7	− 7·0	−1·0
0	14·8	107·2	13·7	0·8			
4·0	17·6	104·0	21·6	1·1	− 3·2	+ 7·9	+0·3
0	17·5	83·9	32·6	1·2			
4·2	—	88·7	34·9	1·4	+ 4·8	+ 2·3	+0·2
*0	22·2	49·0	70·0	1·8			
4·4	22·7	61·5	62·2	1·5	+12·5	− 7·8	−0·3
0	22·2	60·5	66·1	0·8			
6·8	21·3	76·1	49·4	1·0	+15·6	−16·7	+0·2
0	21·4	67·0	56·7	0·8			
7·0	20·9	71·2	47·7	1·0	+4·2	− 9·0	+0·2

to extrude lithium very slowly. But many further experiments would be needed in order to arrive at an accurate quantitative estimate of the extent to which active transport of lithium can occur.

The effect of lithium on the electrical activity of frog muscle

Resting and action potentials were recorded with micro-electrodes by using methods and precautions similar to those described by Adrian (1956). The first point to be established was that there was no significant change in the resting potential on exposure to Li for periods of 15–30 min. Thus in one muscle the average resting potential was initially 92·6 mV (S.E. ± 1·4 mV for 13 fibres) in normal Ringer's solution; after 15 min in Li it was 91·5 ± 2·0 mV (8 fibres); and on restoring normal Ringer's solution it was 92·5 ± 1·4 mV (11 fibres). Another muscle gave very similar results. The action potential was equally unaffected, the average spike heights (for 3 fibres in each case) being 122 ± 3 (in Na), 119 ± 1 (in Li), and 122 ± 2 mV (in Na). The recordings reproduced in Fig. 2 show that there was also no obvious change in the rate of rise of the spike, whose shape was virtually identical in the three cases.

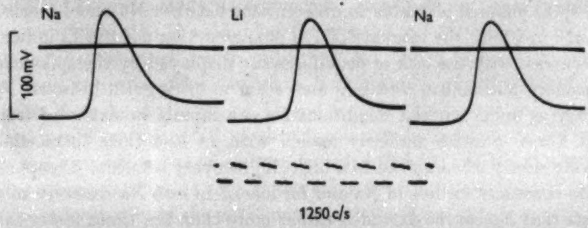

Fig. 2. Resting and action potentials recorded (*a*) in normal Ringer's solution, (*b*) after 20 min in Li Ringer's solution, (*c*) after restoring normal external [Na]. Each record is from a different fibre in the same muscle. Temperature 21° C.

In view of the small number of muscles examined, and the appreciable variability between individual fibres, these observations do not exclude the possibility that there are immediate *slight* changes in the resting and action potentials. But they suggest that any such changes are not likely to exceed 1–2 mV. It also remains possible that larger changes may occur after the internal [Na] has fallen lower than it was given time to do in these experiments, which did not extend beyond a period of 30 min in Li Ringer's solution.

DISCUSSION

The main conclusion to be drawn from the work described here is that there is a clear difference in selectivity between what may be termed the 'spike' and 'recovery' channels for transport of ions through the cell membrane. In agreement with observations on squid axons (Hodgkin & Katz, 1949), isolated frog nerve fibres (Huxley & Stämpfli, 1951) and other excitable tissues (further references are given by Schou, 1957), there is very little immediate change in the electrical activity of frog muscle fibres when Li is substituted for Na in the external medium. This implies that the 'spike' channel, that is to say the passive permeability mechanism which in the resting state allows only a slow trickle of Na^+ ions to flow down the concentration gradient into the interior of the cell, but which permits a greatly accelerated entry during the rising phase of the spike (Hodgkin & Horowicz, 1959), hardly discriminates at all between Na and Li. In the preceding paper (Keynes & Swan, 1959) it has been seen that roughly half the total Na influx of 4·2 pmole/cm^2.sec probably arises from an exchange diffusion process, leaving an influx of some 2 pmole/cm^2.sec reasonably attributable to a passive entry of Na via the spike channel. If the spike channel is unable to differentiate between Na and Li, this figure fits fairly well with the estimate of 1–2 pmole/cm^2.sec for the Li influx.

In contrast with the lack of discrimination displayed by the spike channel, the recovery mechanism certainly does seem to distinguish between Na and Li. Whereas under suitable conditions Na can rapidly be extruded from Na-loaded fibres, muscles similarly loaded with Li lose their intracellular Li relatively slowly when soaked in a high [K] recovery solution. Comparison of the rate constants for loss of Na and for loss of Li into Na recovery solutions suggests that Na can be extruded rather more than ten times faster than Li, but this estimate probably errs in favour of Li, since at least part of the Li which emerges into these solutions seems to do so through the operation of a Li/Na exchange process rather than being transported by a Li pump coupled with an uptake of K. Another method of deriving a figure for the rate at which Li can be extruded is to suppose that in the first five pairs of muscles in Table 7 the Li efflux was exactly keeping pace with the Li influx, and that the influx was the same (say 2 pmole/cm^2.sec) as that measured in fresh muscles. On this basis the rate of extrusion of Li would have been about 3 m-mole/kg.hr from muscles whose average Li content was 77 m-mole/kg, corresponding to a rate constant of 0·04 hr^{-1}. As has already been mentioned (see p. 630) the rate constant for extrusion of Na under equivalent circumstances is slightly over 1 hr^{-1}, so that the relative efficacy with which the recovery channel handles Na and Li would come out in the region of 25/1. There are indications (see, for example, Hodgkin & Horowicz, 1959) that the Na influx tends to increase with time after dissection; if the Li influx had behaved similarly, the muscles of Fig. 7 would have been both gaining and extruding lithium somewhat faster than has just been assumed. Although the argument is complicated by the possibility that under the conditions of Table 7 the passive efflux of lithium via the spike channel was not negligible, it seems fair to regard 25/1 as an upper limit and 10/1 as a lower limit for the relative efficacy of extruding Na and Li.

The behaviour of the muscle membrane towards Li seems similar to that of the erythrocyte membrane, since Maizels (1954) showed that in human red cells the inward passive movements of Na and Li had roughly equal rate constants, but that there was little or no active outward movement of Li. Another tissue where active transport of Li has recently been examined is the isolated frog skin. Zerahn (1955) found that when the skin was bathed with Ringer's solution in which 80% of the Na was replaced by Li the short-circuit current soon fell to about a tenth of its value in normal Ringer's solution, at which level it could apparently be maintained for some while; at the same time, appreciable amounts of Li accumulated in the skin. He established that some active transport of Li was taking place from one side of the skin to the other, and although precise values for the relative affinity of the mechanism for Na and for Li cannot easily be derived, his results do not seem inconsistent with our evidence that the ratio lies between 10 and 25 times. If in general Li enters cells not much less readily than Na, but is extruded much more slowly, then on prolonged exposure to Li one would expect much of the intracellular K to be displaced by Li and in excitable tissues the membrane potential would gradually fall. A slow depolarization in Li of various types of muscle and nerve fibre has often been recorded (Schou, 1957), and a tendency for Li to accumulate inside cells at the expense of K would account satisfactorily for at least part of the well-known toxicity of the ion.

If mammalian C fibres are stimulated in Li solutions, they give normal action potentials (at any rate for short periods), but the post-tetanic hyperpolarization and positive after-potential are immediately abolished (Ritchie & Straub, 1957; Greengard & Straub, 1958). On the assumptions that, as in muscle, the Li that enters the fibres during the impulse is not quickly extruded afterwards, and that consequently there is in Li no enhanced uptake of K after passage of an impulse, these observations have been used by their authors to support interesting hypotheses as to the origins of the hyperpolarization and after-potential. Thus the differences in the behaviour of excitable membranes towards Na and Li have already proved to be of analytical value.

SUMMARY

1. Two methods of determining the size of the lithium influx into frog muscle fibres gave values of 1–2 pmole/cm^2.sec.

2. When Li-loaded muscles were soaked in a sodium recovery medium containing 10 mM-K they lost their Li with a rate constant just under 0·1 hr^{-1}, this figure being about one tenth of that observed by Desmedt (1953) for the extrusion of Na into the same solution.

3. When K was omitted from the recovery medium loss of Li still took place at the same rate, suggesting that efflux of Li into a sodium solution is

not obligatorily linked to an influx of K. It was verified that extrusion of Na from Na-loaded muscles was effectively blocked in a K-free medium.

4. When Li-loaded muscles were soaked in a lithium recovery medium containing 10 mM-K, their Li content remained stationary for about 4 hr, and then began to increase.

5. Micro-electrode recordings showed that there was very little immediate change in either the resting or the action potential when Li was substituted for Na in the external medium.

6. It is concluded that the passive permeability mechanism responsible for generating the action potential does not discriminate between Na and Li, but that the active transport mechanism is only capable of extruding Li from the interior of muscle fibres 1/10–1/25 as fast as it extrudes Na.

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OBSERVATIONS ON AMINE OXIDASE IN HUMAN SALIVARY GLANDS

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Much work has been done on the activity of the enzyme amine oxidase, the catalyst of the biological inactivation of many amines of pharmacological interest. Little, however, is known about its distribution in human tissues. Advantage was therefore taken of the opportunity of examining the salivary glands, submaxillary as well as parotid, obtained fresh after surgical removal of the glands for various reasons.

METHODS

The tissue was removed because of a tumour in or near the gland. Only apparently healthy glandular tissue was used for the measurement of the enzymic activity. The tissue was at once frozen and kept at −20° C. It was later thawed and homogenized in a glass homogenizer with 0·067 M-Na-phosphate buffer of pH 7·4. The amount of tissue from each case was 1–2 g; the total volume was brought up to 10 ml. by adding buffer solution after homogenization.

For the manometric determination of enzymic activity 1·0 ml. of the extract was used in the earlier experiments; these were carried out in conical flasks of conventional size, with a total reaction volume of 3·0 ml. In the later work smaller flasks were used and the amount of homogenate was reduced to 0·4 ml., with a total reaction volume of 0·7 ml. Substrate concentration was 10^{-2} M. Carbon dioxide was absorbed by N-KOH. In experiments with cyanide, KOH was replaced by N-KCN. The gas phase was O_2 and the temperature was 37·5° C.

When inhibitors of amine oxidase were tested they were present in the main compartment of the flask together with the enzyme before the start of the enzymic reaction. Unless otherwise stated, tyramine was used as substrate in inhibitor experiments. In experiments with iproniazid the enzyme was always incubated for at least half an hour at 37·5° C before the addition of the substrate.

RESULTS

Amine oxidase activity of normal human salivary glands

Four submaxillary glands and seven parotids were tested for amine oxidase activity. Table 1 shows the oxygen consumption (μl. O_2/hr/g tissue) using tyramine as substrate. These rates have been calculated from the initial 15 min period of observation. In most of the experiments the rate of the enzymic oxidation during this period was linear and the enzyme blank was very small.

The data given in Table 1 show that the enzymic activity of the homogenate was very high. The mean rate of oxygen consumption in submaxillaries was 1830 μl. O_2/hr/g and in parotids 1000. In most mammalian species examined the liver has been found to be the organ with the highest activity. From the report by Blaschko, Richter & Schlossmann (1937) the activity in guinea-pig liver can be calculated to about 485 and in rat liver to about 265 μl. O_2/hr/g, i.e. considerably lower than the figures found for human salivary glands. The figures for activity of human glands are also much higher than those of cat glands; these were 206 for the submaxillaries and 105 for the parotids (Strömblad, 1956). In other words the enzymic activity of the human glands is about 10 times those found for the cat.

TABLE 1. Enzymic activity expressed in μl. O_2/hr per gram of tissue in human parotid and submaxillary glands. The subjects are arranged according to age, the oldest at the bottom

	Parotid		Submaxillary	
Subject	Male	Female	Male	Female
L.J.	—	1030	—	—
A.R.	1020	—	—	—
V.S.	—	1110	—	—
G.L.	800	—	—	—
D.A.	—	1120	—	—
V.C.	—	—	1450	—
H.C.	—	—	—	2850
A.J.	—	—	1250	—
A.J.	1220	—	—	—
A.P.	—	—	1760	—
J.A.	—	700	—	—

Inhibitor specificity

The intracellular amine oxidase of mammalian tissues is known to be insensitive to cyanide and carbonyl reagents, but is known to be inhibited by octyl alcohol, pentamidine, methylene blue, and the isopropyl derivative of isonicotinic hydrazide (Marsilid, Roche Products; iproniazid). The enzyme of the human salivary glands behaved in all these respects as a typical amine oxidase. With 10^{-3} N cyanide there was some reduction of the rate of uptake of oxygen and with semicarbazide a similar reduction occurred. It is known that these substances reduce the oxygen consumption by inhibiting further oxidation of the aldehyde formed in the primary oxidation reaction. In one experiment with 1 ml. of extract 55 μl. of oxygen was consumed in 15 min; in the presence of 10^{-3} N cyanide the corresponding figure was 39 μl., i.e. a reduction by 29 %. In the same experiment 10^{-4} M pentamidine reduced the oxygen uptake to 7 μl. With 10^{-2} M semicarbazide there occurred a reduction of the oxygen consumption by 25 %. With octyl alcohol the enzymic oxidation was almost completely inhibited. Methylene blue 10^{-3} M caused an inhibition by 82%. No inhibition was observed in the presence of aminoguanidine. 10^{-3} M Marsilid caused a complete inhibition of enzymic oxygen uptake.

Substrate specificity

Different amines were tested as possible substrates of the enzyme from human glands. In each experiment the oxidation was compared with that of tyramine, the amine which proved to be most rapidly oxidized. These experiments are summarized in Table 2. Different homogenates were used but since tyramine was always included the activity is given as a percentage of the rate of oxidation of tyramine.

Table 2. **Enzymic oxidation of various substrates by human submaxillary extracts. The oxidation rate is given as a percentage of that of tyramine**

Tyramine	100	β-Phenylethylamine	35
5-HT	70	Noradrenaline	26
Tryptamine	66	Sympatol	26
Adrenaline	60	*n*-Hexylamine	17
*iso*Amylamine	42	Benzylamine	<10

In addition to the compounds listed the following amines were tested but no significant oxygen uptake was found: 1,4-methylhistamine, mescaline, spermine, spermidine, and histamine. With tryptamine and 5-HT there occurred a significant oxygen uptake and as the reaction proceeded a brown coloration of the material was noted. The two aliphatic amines tested, *n*-hexylamine and *iso*amylamine, were oxidized.

In addition to the substrates so far mentioned a sample of metanephrine was tested and found to be oxidized at about 35% of the rate of tyramine. The rate of enzymic oxidation of metanephrine was also compared with those of adrenaline and noradrenaline. Adrenaline was found to be oxidized at a higher rate and noradrenaline at a lower rate than metanephrine. In order to find out how much of the oxygen consumption with adrenaline and noradrenaline was due to oxidation reactions other than that catalysed by amine oxidase, an experiment was carried out in which the enzyme was first incubated for half an hour at 37·5° C with 10^{-3} M Marsilid. It was found that in the first 15 min period after tipping in adrenaline or noradrenaline there was no oxygen consumption in the flasks containing homogenate incubated with Marsilid; this was taken to indicate that the oxygen consumption observed during the first 15 min without Marsilid was due to the action of amine oxidase. The oxygen consumption with metanephrine was also abolished in the presence of Marsilid. Thus it can be concluded that all three substances adrenaline, noradrenaline, and metanephrine, are readily oxidized by the human enzyme.

DISCUSSION

The most interesting outcome of the experiments reported in this paper is the presence of a very highly active amine oxidase in human salivary glands. Figures for amine oxidase activity of man have been given for the kidney by Blaschko (1942), for intestine, liver and kidney by Epps (1945), for placenta by Thompson & Tickner (1949) and for kidneys and liver by Holtz, Heise & Spreyer (1937). Finally, Zeller (1951) quotes figures for various human organs. It can be calculated that the activity in salivary glands is higher than the activity in the other tissues mentioned. The enzymic activity of human salivary glands is high not only in comparison with the other human tissues that have been examined, but also when it is compared with the enzymic activity of the same glands in the cat (Strömblad, 1956).

At the present state of knowledge the significance of the high enzymic activity of these glands in man is not clear. It has been shown that the enzyme will act on adrenaline, noradrenaline, metanephrine, and 5-HT, but the function of these normally occurring amines in relation to the activity of the glands is poorly understood. It is known that these glands receive adrenergic innervation. There is evidence that in man adrenaline (Emmelin & Strömblad, 1954) and sympathetic stimulation (B. Folkow and J. E. Laage-Hellman, personal communication) will promote secretion from the submaxillary gland, but not from the parotid. The findings reported in this paper make it worth while to consider the possibility that the glands inactivate not only amine locally released, but also amine carried to the glands by way of the blood stream. It seems of interest in view of recent work on the metabolism of catechol amines (Axelrod, 1957) that not only adrenaline but also its *o*-methylated analogue, metanephrine, is readily oxidized. Thus, the human enzyme from salivary glands resembles amine oxidase from other mammalian sources (Leeper, Weissbach & Udenfriend, 1958).

There was, however, one difference in the pattern of substrate specificity from other enzymes: 1,4-methylhistamine was not oxidized. It is known that this amine is an important metabolite of histamine also in man (Schayer & Cooper, 1956; Lindell, Nilsson, Schayer & Westling, 1958). The oxidation of this amine in the mouse *in vivo* is brought about by an enzyme resembling amine oxidase (Rothschild & Schayer, 1958), and it has been shown that the amine oxidase of guinea-pig liver will act upon it (Lindell & Westling, 1957). It is of interest therefore that the amine oxidase studied in this paper does not act on this amine.

SUMMARY

1. Human salivary glands were found to contain an amine oxidase of very high activity.

2. The enzyme was found to be a typical intracellular mono-amine oxidase.

3. The enzyme was found to oxidize 5-HT and metanephrine at rates comparable to those of adrenaline and noradrenaline.

I wish to thank the staff of the Departments of Otolaryngology, Universities of Gothenburg and Lund, for supplying salivary glands, Dr B. Witkop, Bethesda, for a gift of metanephrine and Dr H. Blaschko, Oxford, for his generous interest and support of this investigation.

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Manometer requiring minimal movements of fluid. By H. DAVSON and C. PURVIS. *Department of Physiology, University College London*

An instrument has been developed capable of registering changes of pressure in the range 0–100 mm Hg, with a fluid movement of only 0·03 μl./mm Hg. Changes in pressure are transmitted to a beryllium-copper diaphragm, the centre of which is soldered to a twisted phosphor-bronze strip on which is mounted a mirror. Displacement of the diaphragm results in a rotation of the mirror (Fig. 1).

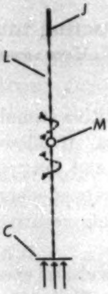

Fig. 1

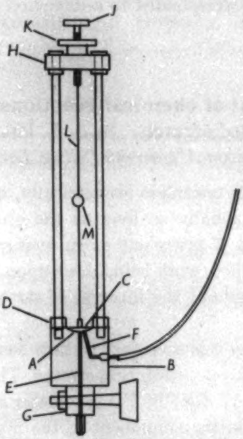

Fig. 2

Fig. 1. Illustrating the mirror, *M*, mounted on the phosphor-bronze strip, *L*. *C* represents the diaphragm. The arrows indicate the directions in which the strip has been twisted; these are opposite, above and below the mirror.

Fig. 2. Illustrating the complete assembly. For details see text.

The instrument is illustrated schematically in Fig. 2. The pressure-chamber, *A*, is a cavity 10 mm in diameter by 1 mm depth, bored out of a block of Perspex, *B*. It is covered by the beryllium-copper diaphragm, *C*, which is held in position by a Perspex washer, *D*, through which pass six screws. The diaphragm is 0·05 mm thick and has four equally spaced annular corrugations. Connexion with the outside is made by two holes, *E* and *F*, bored in the Perspex block; the central one, *E*, may be closed by the tap, *G*; the other hole ends in a steel tube by means of which the chamber may be connected—by way of polythene tubing—to the system whose pressure is to be studied (e.g. the anterior chamber of the eye). Through the tap, *G*, connexion may be made to a reservoir of saline by means of which the chamber and polythene tubing are filled.

The platform, *H*, is supported by two brass rods and holds the adjustment heads, *J* and *K*. The twisted phosphor-bronze strip, *L*, is 0·025 mm thick and 0·037 mm wide; it is connected at one end to a pin, soldered into the centre of the diaphragm, and at the other end to the adjustment head.

Light tension is applied to the strip by rotating *K* with *J* held stationary; *J* and *K* are then locked together, and centring the mirror *M* is carried out by rotating *K*.

With a distance of about 2 m between scale and mirror, a change of pressure of 1 mm Hg corresponded to a deflexion of 4 cm.

The reversal of chemical reactions in contracting muscle during an applied stretch.

By A. V. HILL and J. V. HOWARTH. *Department of Physiology, University College London*

When a muscle contracts isometrically, under repetitive stimulation, it gives out heat continually so long as the stimulus lasts. If allowed to shorten against a load it gives out extra energy as work and heat. Conversely, if forcibly stretched, work being done upon it, its net rate of energy liberation is reduced throughout the interval of stretch, becoming negative with quicker stretches.

For accuracy it is necessary to take account of the elastic energy due to the high tension developed during stretch. The net work W_n is $(W-E)$, where W is the gross work done by the ergometer and E is the elastic energy developed in the series elastic component of the muscle and in the connecting chain or wire. Then the net energy liberated by the active contractile component is $(H-W_n)$, where H is the observed heat production. All these quantities are measured as functions of the time from the beginning of stimulation.

Throughout a rather rapid stretch, e.g. 4 mm in 0·5 sec in a toad sartorius at 0° C, $(H-W_n)$ diminishes considerably: in words, the net rate of energy liberation is substantially negative (numerically about as large as the positive value under isometric conditions). When the stretch stops, the stimulus continuing, $(H-W_n)$ starts to rise again: energy is absorbed only while the contractile component is being stretched. Presumably this energy is used in driving chemical resynthesis.

With an appropriate stretch and stimulus, the total energy $(H-W)$ given out by a muscle in contraction and relaxation may be about nil.

The apparatus will be demonstrated. The heat is recorded by a quick thermopile and galvanometer; the stretch is applied, at constant speed, by a Levin–Wyman ergometer joined to the muscle by a strong chain; the work is calculated from a tension record. The elastic energy in the chain is read off from an experimental curve relating energy to tension, that in the series elastic component from a similar mean curve deduced from the relation, between force and distance, observed when a muscle is rapidly released from an isometric contraction. With good connexions the elastic energy developed is a significant, but not large, fraction of the work done in the stretch: the chief part of the work is taken up directly by the contractile component and there turned presumably into chemical energy and heat.

Method of studying motor conditioned reflexes in rats.

By V. Podachin. *Department of Physiology, University College London*

Spindle motor discharges in the rabbit.

By K. Diete-Spiff and J. E. Pascoe. *Department of Physiology, University College London*

Proprioceptive receptor potentials of oscillatory form. (Film.)

By O. C. J. Lippold, J. Nicholls, B. Payton and J. W. T. Redfearn. *Department of Pharmacology, St Bartholomew's Hospital Medical College, London, E.C.* 1

A method of mounting micro-electrodes for intracellular recording from contracting muscle.

By E. M. Vaughan Williams. *Department of Pharmacology, University of Oxford*

Woodbury & Brady (1956) reported a method of mounting glass microelectrodes upon fine tungsten wire, in order that the point of the electrode would be less easily dislodged from an intracellular position by muscular contractions. It was stated that polarization was not a serious problem. In the author's hands this method had two disadvantages. (1) There was appreciable drift in the electrical base line, even when fine chlorided silver wire was substituted for tungsten. (2) The dangling electrodes were difficult to manipulate into desired positions for repeated recordings from the same region. The following method permitted the use of one carefully matched pair of Ag–AgCl wires for connexion to many electrodes, while allowing free movement of the electrode tip.

Electrodes were drawn from hard glass, O.D. 2 mm, I.D. 1 mm, by an apparatus modified from that described by Alexander & Nastuk (1953). After an electrode had been filled with 3M-KCl, a circular cut on the shaft was made with a diamond (Fig. 1A). The shaft was then dipped, blunt end downwards, into a latex solution, and immediately withdrawn. The latex was allowed to dry in air, while the electrode was held vertically in a special holder which kept the tip in 3M-KCl (Fig. 1B). When dry the part of the latex which formed a cap over the blunt end was cut away. The shaft was then broken at the circular cut, and the two halves of the shaft were slid apart inside the latex sleeve, which maintained a watertight but very light junction between them (Fig. 1C). The blunt end of the shaft could now be fitted into a small polythene tube which connected it via an Agar–Ringer bridge to one member of a pair of Ag–AgCl wires enclosed in a black Perspex shield (Fig. 1D).

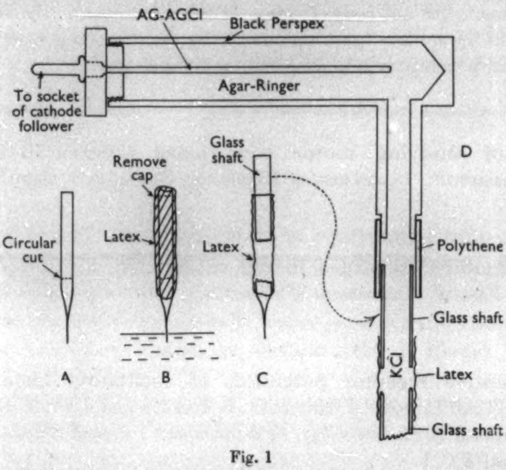
AG-AGCl
Black Perspex
Agar-Ringer
To socket of cathode follower
Glass shaft
Remove cap
D
Circular cut
Latex
Latex
Polythene
Glass shaft
KCl
Latex
Glass shaft
A
B
C

Fig. 1

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An incubating chamber for metabolism studies. By A. K. Huggins, J. Martin and J. H. Ottaway. *Department of Biochemistry, University College London*

The conventional Warburg flask has often been used for incubating small slices of tissues such as rat liver or diaphragm muscle *in vitro* (Ottaway, 1951) but is not very convenient when measurements of gas exchange are not required. The tissue slices may be incubated in a shaker of the Dubnoff type (Dubnoff, 1948) but this has the disadvantage that, when more than one animal is used, all the slices must be placed in the apparatus together, with consequent differences in the time interval between the death of the animal and the start of the incubation period.

The apparatus to be described is convenient for the incubation of slices from a single animal when gas measurements are not required; it can be attached to the conventional supports used for Warburg manometers and shaken in an ordinary Warburg bath. It consists of a rectangular box ($3\frac{1}{2}$ in. × $2\frac{1}{2}$ in. × $1\frac{1}{2}$ in.; $8{\cdot}9 \times 6{\cdot}4 \times 3{\cdot}8$ c.c.) made of $\frac{5}{16}$ in. (8 mm) Perspex sheet, with a detachable, flanged watertight lid. Through the lid pass Perspex tubes used for gassing, which can be closed off by means of two screw clips on rubber connexions. A Perspex tray, drilled with a series of holes, fits inside the chamber. In the holes rest small glass beakers (1 cm diam. × 2 cm high) for the tissue slices. The apparatus shown has a beaker which is used for buffer only, to verify that no evaporation occurs during the experiment. The lid is greased and is held in place by means of a spring clip (Herbert Terry and Sons Ltd, Redditch) at each end. The chamber is attached to a metal support by means of metal slides (as used for Warburg manometers) and a retaining spring. In order to prevent evaporation during gassing, strips of moistened 'Cellosene' wadding are placed round the chamber walls and the gas is pre-saturated with water vapour. A bent glass tube attached to the gas outlet, and dipping under the water in the bath, allows the rate at which gas is passing through the chamber to be observed. The design of the gas inlets and outlet has been arranged to ensure uniform gassing of all the beakers in the tray. Approximately 1 l. of gas is passed in 5 min to ensure a uniform atmosphere inside the chamber. By varying the dimensions and the number of beakers, a variety of *in vitro* metabolism studies not involving measurement of respiratory gas changes can be performed.

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A constant-voltage general purpose transistor stimulator. By

C. A. Joslin. *Department of Pharmacology, Charing Cross Hospital Medical School, London, W.C.* 2

The stimulator circuit illustrated (Fig. 1) is suitable for use where constant voltage output pulses are required. These output pulses are controlled by a potentiometer, and the range is 0–40 V.

The time of rise for the output pulses is less than 10 μsec. They are rectangular in form and the base-line potential is zero volts.

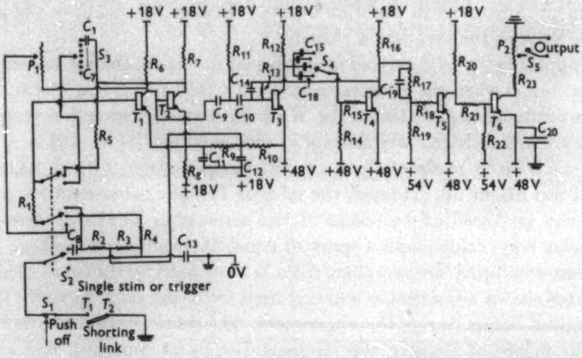
+18V
+18V
+18V
+18V
+18V
+18V
C_1
S_3
P_1
C_7
R_6
R_7
R_{11}
R_{12}
C_{15}
S_4
R_{13}
C_{14}
C_{16}
R_{16}
P_2
Output
S_5
R_{20}
R_{17}
C_{19}
R_{23}
T_1
T_2
C_9
C_{10}
T_3
R_{15}
T_4
R_{18}
T_5
R_{21}
T_6
C_{20}
R_5
R_{14}
R_{19}
R_{22}
R_8
C_{11}
R_9
R_{10}
C_{12}
+48V
+48V
+48V
+ 54V
+ 48V
+ 54V
+ 48V
+18V
+18V
R_1
C_8
R_2
R_3
R_4
C_{13}
0V
S_2
Single stim or trigger
S_1
T_1 T_2
Push off
Shorting link

Fig. 1. Circuit diagram of transistor stimulator. $R_1 = 47$ kΩ, $R_2 = 220$ kΩ, $R_3 = 22$ kΩ, $R_4 = 47$ kΩ, $R_5 = 470$ kΩ, $R_6 = 100$ kΩ, $R_7 = 50$ kΩ, $R_8 = 5{\cdot}8$ kΩ, $R_9 = 3{\cdot}3$ kΩ, $R_{10} = 10$ kΩ, $R_{11} = 470$ Ω, $R_{12} = 68$ kΩ, $R_{13} = 150$ kΩ, $R_{14} = 10$ kΩ, $R_{15} = 470$ Ω, $R_{16} = 4{\cdot}7$ kΩ, $R_{17} = 22$ kΩ, $R_{18} = 47$ Ω, $R_{19} = 62$ kΩ, $R_{20} = 3$ kΩ, $R_{21} = 47$ Ω, $R_{22} = 10$ kΩ, $R_{23} = 47$ Ω; $C_1 = 10$ μF, $C_2 = 1{\cdot}6$ μF, $C_3 = 0{\cdot}6$ μF, $C_4 = 0{\cdot}2$ μF, $C_5 = 0{\cdot}06$ μF, $C_6 = 0{\cdot}012$ μF, $C_7 = 0{\cdot}003$ μF, $C_8 = 0{\cdot}002$ μF, $C_9 = 0{\cdot}05$ μF, $C_{10} = 0{\cdot}1$ μF, $C_{11} = 5$ μF, $C_{12} = 5$ μF, $C_{13} = 0{\cdot}02$μF, $C_{14} = 100$ pF, $C_{15} = 0{\cdot}001$ μF, $C_{16} = 0{\cdot}005$ μF, $C_{17} = 0{\cdot}025$ μF, $C_{18} = 0{\cdot}1$ μF, $C_{19} = 0{\cdot}001$ μF, $C_{20} = 16$ μF, 100 V d.c.; $P_1 = 1$ MΩ, $P_2 = 2$ kΩ (wire-wound). T_1–T_5 = OC76, T_6 = V60/20P.

The pulse repetition frequency is adjustable from 3/min up to 700/sec in seven ranges. These ranges may be extended, either way, by suitable modification.

Four pulse widths, 0·2, 1, 3 and 14 msec can be selected by switching and it is intended that the full range of intermediate values shall be made adjustable in future models.

Provision is made for the pulses to be either repetitive or single. When single pulses are required they may be produced by internal or external switching, or alternatively by electrical triggering, the latter facility being useful where it is necessary to drive several circuits, including the stimulator, from a common source.

Basically the circuit consists of a heavy-duty output transistor (T_6) which is driven by pulses derived from a univibrator type of circuit. In order that sufficient drive power can be supplied to the output transistor, a buffer amplifier (T_5) is included. The univibrator (T_3T_4) is in turn triggered by pulses derived from an asymmetrical multivibrator circuit (T_1T_2). The latter circuit is extremely stable, even within reasonable variations of temperature.

Power may be supplied by a suitably tapped high tension battery or mains power unit. The mean current drain is 10 mA when the output is unloaded. When the output is heavily loaded, at 500Ω (say), the current pulses taken from the battery approach 80 mA. However, the mean current taken from the battery is still fairly low.

A battery power supply limits the constant voltage characteristic of this stimulator. With a suitable mains power unit the output pulses will only vary slightly in amplitude when load changes from infinity down to 500Ω are applied.

A 'balanced closed circuit' spirometer of low resistance. By I. P. Priban. *Institute of Neurology, The National Hospital, Queen Square, London, W.C.* 1

The disadvantages encountered in the conventional Knipping type spirometer circuit have been reduced. In the system demonstrated the subject breathes into the circuit at a point which is at atmospheric pressure, the ducting has a low resistance and the recording spirometer used is a new type that was described by Bernstein, D'Silva & Mendel (1952).

The air flow produced by two centrifugal fans is balanced so that the point in the circuit into which the subject breathes (A) and the spirometer bell (B) are at atmospheric pressure (see Fig. 1). This is done by adjusting the air flow at one or more resistances (see points R_1 to R_5 in Fig. 1) in the circuit, while it is open to atmosphere at A, until there is no movement of the recording bell at the middle position. The magnitude of resistance required for balancing does not reduce the air flow below 175 l./min. By working one fan at a time in the completely closed circuit, or in the spirometer-patient closed circuit, pressure gradients with respect to atmosphere are produced. These permit easy detection of leaks.

A large CO_2 absorbing canister (cross-sectional area 175 cm^2 and height 23 cm) is used for its low air-flow resistance. Furthermore, it is only filled $\frac{1}{3}$ to $\frac{2}{5}$ with soda-lime; thus the expanded cross-sectional area ensures that pressures are minimal so that soda-lime dust is not carried around the circuit and inhaled. The expired CO_2 of a subject can be allowed to accumulate by by-passing the soda-lime canister through a parallel circuit which is controlled by two three-way taps.

The balanced circuit has a total resistance of approximately 0·5 cm water/l. air/sec at maximum flow velocity. In the circuit the smallest bore is 3·2 cm in the three-way taps, the corrugated tubing has a bore of 3·5 cm; most of the ducting has a bore of 4·5 cm or greater, especially at bends. The total volume of the circuit with the recording bell at zero is about 10 l. The advantages of the recording spirometer are discussed by Bernstein *et al.* (1952) and Bernstein (1954).

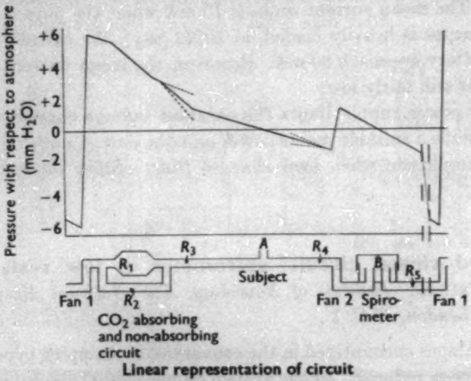
Pressure with respect to atmosphere (mm H_2O)
+6
+4
+2
0
-2
-4
-6
R_3
A
R_4
R_1
Subject
B
R_5
Fan 1
R_2
Fan 2
Spirometer
Fan 1
CO_2 absorbing and non-absorbing circuit
Linear representation of circuit

Fig. 1. Diagrammatic representation of static pressures throughout the 'balanced closed circuit' spirometer.

The system described has been found suitable for measuring ventilatory changes in unconscious human subjects under varying conditions.

I wish to thank British Oxygen Gases Limited for the help received in developing this apparatus.

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A pharmacologically active choline ester and other substances in the Garden Tiger Moth, *Arctia caja* (L.). By M. Schachter.

Department of Physiology, University College London

Femoral mechanoreceptors in the locust. By S. J. Hubbard.

Department of Physiology, University College London

Studies of the function of mechanoreceptors, in which the receptor potential has been established as a precursor of the nerve impulse, have been made in amphibian, mammalian and crustacean receptors, but intracellular recording was possible only in crustacean preparations (Kuffler, 1954). Thus it seemed worth while to exploit the peripheral sensory neuronal organization of the arthropods by investigating receptors in the highly developed sub-phylum of insects. These have had more than their fair share, perhaps, of behavioural studies and the amount of electrophysiological evidence of the function of discrete receptors is relatively small. Pringle (1938) has described a form of mechanoreceptor—the campaniform sensilla—in the cockroach and Finlayson & Lowenstein (1958) have recently reported work on abdominal stretch receptors in insects. This demonstration gives a technique for studying femoral chordotonal organs in the locust, of which a brief anatomical description has been given by Slifer (1935). The preparation has the advantage of being of macroscopic size and of not being intimately associated with muscle fibres.

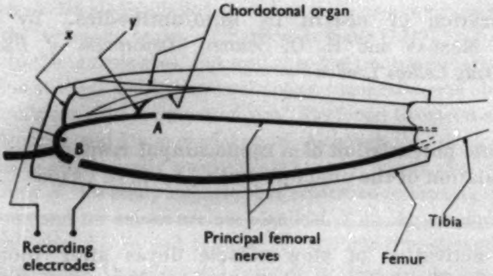

Fig. 1. Diagram showing the nerve supply to the femoral chordotonal organ in the locust. The two principal femoral nerves were cut at *A* and *B*, and the external recording electrodes placed as indicated. x and y are the nerve branches from the two parts of the organ.

The locusts used were female specimens of either the desert locust or the African migratory locust. They were decapitated and a mesothoracic leg was cut off as close to the thorax as possible; the tibia and the remainder of the leg was then cut off about 2–3 mm from the femur. All subsequent dissection was performed under saline of the composition given by Hoyle (1953). A flap of the femoral wall was removed, all extraneous muscle was cut away and the two principal divisions of the femoral nerve cut at *A* and *B* (Fig. 1), taking care to avoid damage to the small branches x, y, from both parts of the organ. The preparation was placed on a layer of beeswax in a cell filled with saline and constructed on a microscope slide. A pair of platinum external recording electrodes were mounted in the cell and connected as shown in Fig. 1 after final cleaning of the nerve. When recording, the liquid in the cell was removed and replaced by liquid paraffin of pharmaceutical purity. The preparation was stretched by a thread attached to the small remaining piece of tibia and a linear transducer was incorporated so as to give a continuous record of length.

Preliminary experiments suggest that the two parts of the chordotonal organ have widely differing properties of threshold and adaptation.

Acknowledgements are due to the Anti-Locust Research Centre, Princes Gate, for providing adequate supplies of locusts and to Dr P. T. Haskell for advice at the start of this work.

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Immobilization of sperm by auto-antibodies.

By J. Baum, J. L. Mongar and H. O. Schild. *Department of Pharmacology, University College London*

Post-tetanic potentiation of a hippocampal response to electrical stimulation of the olfactory bulb.

By B. G. Cragg

Selective activation of slow-muscle fibres after motor nerve section.

By B. Katz and R. Miledi. *Department of Biophysics, University College London*

During experiments on degenerative changes at the myoneural junction we found that, in a given frog muscle (iliofibularis or ext. l. dig. IV), transmission from large motor axons to 'twitch-fibres' fails about two days sooner than transmission from small axons to 'slow-muscle fibres'. Thus, by previous nerve section, it is possible to obtain nerve-muscle preparations whose indirect response consists entirely of slow, non-propagated contractions associated with 'slow-junction potentials'.

Spontaneous activity and structural changes at denervated end-plates of the frog.

By R. Birks, B. Katz and R. Miledi. *Department of Biophysics, University College London*

Prism flame photometer.

By E. J. Harris. *Department of Biophysics, University College London*

On central regulation of rumination and reticulo-ruminal motility.

By B. Andersson, R. Kitchell and N. Persson. *Department of Physiology, Kungl. Veterinärhögskolan, Stockholm* 51

Electrical stimulation at different sites in the medulla oblongata was performed in conscious goats which had previously been supplied with closed ruminal, reticular and abomasal fistulae. Hess's technique (1949) was used with some modifications. The parameters of stimulation were 0·01–0·03 mA, 10–50 c/s, pulse duration 1 msec. The resistance with the electrodes *in situ* was 5–10 kΩ.

In the caudal part of the medulla oblongata, within an area of the formatio reticularis grisea located ventral and lateral to the tractus solitarius, stimulation was seen to produce retching and occasional vomiting. More pronounced vomiting without prior retching was elicited when stimulations were made dorsal to the tractus solitarius at the lateral margin of the area postrema. The area in which electrical stimulation caused retching and vomiting corresponds roughly to the 'vomiting centre' of Borison & Wang (1949).

Adjacent to the 'emetic area' but medial to the tractus solitarius, between it and the dorsal vagal and hypoglossal nuclei, rumination was obtained as a result of stimulation. The 'rumination area' was found to extend about 2 mm rostral to the obex and 1 mm caudal to it. Although the 'rumination area' was found to be anatomically closely related to the 'emetic area' in the goat, several features of this study indicate that central co-ordinating mechanisms for rumination and for emesis are not identical. Thus, for example, at a site where a weak stimulus caused rumination, the stimulus strength could be increased three times, the effect still being to produce rumination. Lowering the strength of stimulation at a site where emesis was obtained only reduced the emetic response but was never seen to convert it into rumination.

Throughout the periods of stimulation within the 'rumination area' (adjacent to or even in the lateral part of the dorsal vagal nucleus) a complete arrest of reticular and ruminal contractions was seen, immediately followed by abnormally intense reticulo-ruminal motility after cessation of stimulation. This observation supports the view that the dorsal vagal nucleus is the 'pacemaker' for reticulo-ruminal motility, since it seems likely that stimulation in the vicinity has blocked rhythmic activity normally existing in the nucleus. A rebound phenomenon would then be a possible explanation of the overactivity seen on cessation of stimulation.

Increased reticulo-ruminal motility as a direct effect of stimulation was only seen during stimulation at sites located within the formatio reticularis alba, i.e. the area medial to the root filaments of the hypoglossal nerve and dorsal to the inferior olivary nucleus.

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Thalamic units responding to cooling of the cat's tongue. By

S. Landgren. *Department of Physiology, Kungl. Veterinärhögskolan, Stockholm* 51

Thanks to the work of Zotterman (1936) and Hensel & Zotterman (1951) the response of the peripheral neurone to cooling of the cat's tongue is known in great detail. It was therefore of interest to record the response to cooling from central neurones in the ascending path and to compare peripheral and central responses. In this investigation the activity of single units in nucleus ventralis posteromedialis of the thalamus was studied. The activity was recorded with KCl-filled micro-electrodes placed within the nucleus with a stereotaxic technique (cf. Appelberg & Landgren, 1958). *Encephale isolé* preparations of cats anaesthetized with ether during the operation were used for the experiments. Surgery was restricted to a minimum and wounds were locally anaesthetized. None of the stimuli used caused movements of the jaws or tongue of a type indicating that the animal was suffering pain. The following stimuli were applied to the tongue:

(1) Touch, pressure and stretch.
(2) Cooling and warming.
(3) Taste solutions (salt, bitter, acid and water).

Cooling and warming was induced by flooding the tongue surface with Ringer's solution of known temperatures or, when the stability of the unit allowed, with a thermode according to the technique of Hensel, Ström & Zotterman (1951).

The effect of electrical stimulation of the tongue and of the ipsilateral dorsal ascending trigeminal tract was also investigated.

Out of sixty-two fully investigated units twenty were found to respond only to cooling of the tongue, four units responded both to touch and cooling of the tongue and one to warming only. Thalamic 'cold cells' were often found close to units responding to touch of the tongue.

Most thalamic 'cold cells' were discharged by electrical stimulation of the tongue with a latency between 6 and 14 msec, which is approximately two to three times as long as the corresponding latency of the 'touch cells'. The latency of their response to electrical stimulation of the tract was 1·0–1·9 msec. Thalamic 'touch cells' showed similar latencies in response to tract stimulation.

Some thalamic 'cold cells' were very sensitive to cooling of the tongue and responded readily to a drop of cold Ringer's solution or to blowing a puff of air over the tongue surface.

Seven units were tested with the thermode technique. A drop in temperature of approximately 5, 10 or 15° C was applied to the tongue. The temperature was kept constant before and after the change. The thalamic units generally responded to this test in a way similar to that of the peripheral unit, i.e. with an increased frequency of discharge, which adapted to a new and higher constant level. Warming the tongue reduced or stopped the thalamic discharge. An obvious relationship was observed between the intensity of the thermal stimulation and the discharge of the thalamic 'cold cell'.

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The effect of neuronal rest on the output of sympathetic transmitter from the spleen of the cat.

By G. L. Brown, B. N. Davies* and C. B. Ferry.* *Department of Physiology, University College London*

Brown & Gillespie (1957) have shown that the amount of transmitter appearing in the venous blood from the spleen is related to the frequency at which the post-ganglionic sympathetic nerves are stimulated. At 30/sec about 1000 pg per stimulus can be collected, whereas at 10/sec the amount falls to about 200 pg. The output at 10/sec can be made to equal that at 30/sec by giving a blocking agent like dibenzyline (*N*-phenoxyisopropyl-*N*-benzyl-β-chloroethylamine). In their experiments the post-ganglionic nerves were stimulated about 1 hr after they had been tied and 10 min after an 'emptying stimulus' had been given when the blood was not collected. In the present experiments, we have 'rested' the post-ganglionic neurones by more or less complete section of the preganglionic neurones 24 hr–6 days previously. This was done by dividing under ether anaesthesia and aseptic conditions the left superior splanchnic nerves; in the cat the left nerves supply the cells of origin of five-sixths of the post-ganglionic fibres supplying the spleen. In the final experiment, the post-ganglionic nerves were tied as soon as the cat was anaesthetized with chloralose, and they were stimulated with groups of 200 maximal shocks at a frequency of 30/sec. In nine experiments the mean output in response to the first stimulation was 293 pg/stimulus (S.E. 42). If the stimulation was repeated at 10 min intervals, the output per stimulus increased each time until about 1000 pg/stimulus was reached. A similar, but immediate, increase in output was produced by giving 1000 conditioning stimuli at 30/sec 10 min before the standard test stimulus of 200 shocks. The low output of transmitter after neuronal rest could be due either to deficient liberation at the nerve terminals or to increased inactivation of the liberated transmitter before it reached the venous effluent. Administration of dibenzyline raises the output from the rested neurones to the normal level, and after dibenzyline the 'conditioning' train is without effect.

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* M.R.C. Scholar.

It is suggested that the amount of transmitter appearing in the venous blood is dependent upon the number of receptors already occupied by transmitter; resting the neurones increases the number of vacant receptors available for combination with the transmitter.

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The effect of reserpine on the electrical activity in preganglionic sympathetic fibres.

By A. Iggo and Marthe Vogt. *Departments of Physiology and Pharmacology, University of Edinburgh*

Reserpine causes a loss of noradrenaline and of 5-hydroxytryptamine from the brain, and of noradrenaline from the peripheral adrenergic neurones. The loss of transmitter may be sufficient to mimic sympathectomy. Much of the cerebral noradrenaline and 5-hydroxytryptamine is localized in the sympathetic centres; to see whether the brain, which had been depleted of these amines, was still discharging normally into the preganglionic sympathetic fibres, we compared preganglionic activity in normal and in reserpine-treated cats. Any abnormality found after administering reserpine might give a clue to the functional role of these amines.

Preganglionic impulses in fine strands of the cervical sympathetic nerve were recorded in cats anaesthetized with chloralose.

Normal cats. Several patterns of activity were found (cf. Adrian, Bronk & Phillips, 1932): (1) activity which was inhibited by pressor doses of adrenaline; (2) discharges brought on by acoustic stimuli; (3) discharges showing a respiratory rhythm which was sometimes dissociated from respiratory blood pressure fluctuations. Asphyxia greatly enhanced frequency of discharge and number of active units. Morphine, injected intravenously in large doses (2–7 mg/kg), caused a great increase in efferent activity which started before the deep fall in blood pressure that followed the injection.

The reserpine-treated cats were given daily intraperitoneal injections of reserpine (1 mg/kg) for 4 days and were used on the fifth day. Hypothalamic noradrenaline fell to $< 7\%$ of normal after the same treatment. The efferent sympathetic activity in the undisturbed state (blood pressure mean 90 mm Hg) was certainly no less than normal and was usually more continuous. As in normal cats, it was inhibited by adrenaline and stimulated by noise and by asphyxia; the activation by morphine was much reduced. The stimulation by asphyxia was slow in onset and was, as expected, accompanied by a fall instead of by a rise in blood pressure.

The conclusion is that reserpine did not inhibit the discharge of impulses into preganglionic sympathetic fibres. The greater regularity of firing might have been due to the unsuccessful attempt of the centres to restore the blood pressure by vasomotor activity. It would appear that the noradrenaline and 5-hydroxytryptamine content of the brain bears no relation to the sympathetic outflow from the brain, and that these substances are either not essential, or only required in minute amounts, for normal central sympathetic activity.

Bein (1955) has reported the complete absence of activity in the nervi accelerantes of cats 1 hr after small doses of reserpine. Under similar conditions we observed in cervical sympathetic fibres only a slight decrease in activity followed by a return to or above the resting level.

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Non-homogeneity in muscle slices. By R. Creese. *Department of Physiology, London Hospital Medical College, London, E.* 1

Anoxia produces differences in function between various layers of isolated muscle, the superficial fibres showing little change in resting potential while the fibres in the depth of the tissue become depolarized (Creese, Scholes & Whalen, 1958). Experiments were made to detect changes in the potassium content of different layers in these conditions.

Diaphragm muscles from rats weighing 160–180 g were soaked in saline containing ^{42}K (Creese, Neil & Stephenson, 1956), a gas mixture of 95% oxygen and 5% carbon dioxide being bubbled through the solution. After 4 hr, mixing of ^{42}K and ^{39}K was complete. In some cases a gas mixture containing 5% oxygen, 5% carbon dioxide and 90% nitrogen was then bubbled through the saline for a further 4 hr. Muscles were removed, the edges were cut away and the flat diaphragm was sliced horizontally by means of a freezing microtome which was set to give sections of 50 μ. The fragments of tissue were dried, weighed and counted, and the results were expressed as counts/min. mg.

In control experiments without anoxia, the ratio middle slice: outer slice (pleural surface) was 101% ± 4·4 (s.e. of eleven; range 81–122). After anoxia the ratio was 44% ± 4·4 (10; $P < 0{\cdot}01$). This is taken to indicate that anoxia depletes the deeper layers of potassium as compared with the superficial layers.

In some situations it is likely that the deeper fibres exchange potassium more slowly than the superficial layers owing to the retarding effect of intercellular diffusion (Harris & Burn, 1949; Keynes, 1954). Muscles were soaked in inactive saline (with 95% oxygen and 5% carbon dioxide) and then transferred to saline containing ^{42}K. After 20 min the muscles were removed and sliced. The ratio middle slice:outer slice was $69\% \pm 4{\cdot}0$ (10), and the difference is significant ($P < 0{\cdot}01$ by t test). Other muscles were soaked for 4 hr in saline containing ^{42}K and then placed for known times in inactive flowing saline. The slices were weighed and counted as usual and the potassium content was determined in some cases. The results give some support to the view that in this kind of experiment the rate of exchange of the whole tissue may be slower than that of the superficial fibres.

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The influence of anions on impulse generation and membrane conductance in Purkinje and myocardial fibres.

By O. F. Hutter and D. Noble.* *Department of Physiology, University College London*

Recent experiments on skeletal muscle have shown that a large proportion of the current flowing through the resting membrane may be carried by chloride ions (Hodgkin & Horowicz, 1957; Padsha, 1957; Hutter & Padsha, 1959). Some information about the role of anions in cardiac muscle has therefore become desirable.

Purkinje fibres were taken from dogs' and sheep's hearts. In preparations beating spontaneously, the frequency could be greatly influenced by replacement of chloride with other anions. The immediate effect of nitrate was a reduction in frequency by about 80%, caused by a decrease in the rate of rise of the pace-maker potential and usually accompanied by some hyperpolarization in diastole. Continued exposure to nitrate led to depolarization and acceleration to about twice the original frequency in chloride. Exposure to bromide has qualitatively a similar effect; iodide nearly always arrested the preparation, recovery only occurring on return to chloride.

These changes could be explained by supposing that Purkinje fibres are more permeable to bromide, nitrate and iodide than to chloride ions, and that the anion equilibrium potential is appreciably less than the maximum diastolic potential. The sudden appearance of these ions in the extracellular fluid might then be expected to suppress the pace-maker potential and hyperpolarize the membrane, but as intracellular chloride is replaced the membrane potential in diastole would be accelerated in its fall towards the anion equilibrium potential, thus promoting pace-maker activity. In the case of iodide we may suppose that the increase in anion conductance is great enough to hold the membrane potential at the anion equilibrium potential, thus arresting the preparation.

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Several additional observations support this interpretation. (i) Replacement of chloride by large monovalent anions, e.g. methylsulphate or pyroglutamate (Boistel & Fatt, 1958) caused an initial acceleration followed by slowing to a lower frequency than in chloride, i.e. the opposite effect to nitrate. (ii) In driven Purkinje fibres or myocardial fibres of cats and rabbits the duration of the action potential is reduced by nitrate and iodide and prolonged by methylsulphate and pyroglutamate. (iii) Under comparable conditions the membrane resistance of Purkinje and myocardial fibres showed a fall in iodide and nitrate and a rise in methylsulphate and pyroglutamate.

By comparison with skeletal muscle the increase in membrane resistance produced by replacing chloride with large anions is small, so that we may assume that in cardiac muscle chloride contributes only a small share to the membrane conductance. This would make the membrane potential less stable in diastole and allow quite small changes in cation permeability to determine the time course of the action potential.

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Corticofugal projections from temporal lobe in cat and their possible role in acoustic discrimination.

By J. E. Desmedt and K. Mechelse. *Laboratoire de Pathologie générale, University of Brussels, Belgium, and Laboratorium voor Neuroanatomie, Ryksuniversiteit, Leiden, Holland*

The organization of corticofugal projections from auditory areas and surrounding cortex has been investigated with the Nauta–Gygax (1954) silver method. Small lesions restricted to the cerebral cortex were made by subpial suction in fourteen adult cats which were killed 7 days later. Serial sections were prepared from the fixed brain, and degenerating terminal axons were looked for. A number of fibre connexions were traced in this way between temporal areas and regions in diencephalon, mesencephalon and pons (to be published in *Arch. ital. Biol.*).

Special emphasis is to be placed on the pattern of corticofugal projections terminating in the caudal diencephalon. After a lesion in the auditory I area, marked terminal degeneration is observed at that level in the medial geniculate nucleus. By contrast, after a lesion situated near the pseudosylvian fissure, the main degeneration occurs in a region situated between the medial geniculate nucleus and the medial lemniscus. Now this very region has recently been identified as a link in a specific descending pathway which, when stimulated, suppresses cochlear nucleus response to sound (Desmedt & Mechelse, 1958). It so appears that one of the main starting points of this centrifugal system is located, not in the classical acoustic projection cortex, but in the anterior sylvian cortex. Moreover, since no corticofugal axons have been found to terminate in the cochlear nucleus itself, we infer that the inhibitory pathway must include at least one synapse.

Suggestive correlates can be drawn from recent psychophysiological evidence showing that bilateral ablation of cortex lying ventral with respect to auditory areas I and II in cat (Goldberg, Diamond & Neff, 1957; Neff, W. D., personal communication) and dog (Konorski, J., personal communication) destroys the ability to discriminate between different sequences of two tones, but preserves simple frequency differentiations. Such a peculiar deficit for pattern perception is not observed after ablation of the dorsal acoustic projection field (Diamond & Neff, 1957). In view of our evidence that ventral temporal cortex in cat is at least partially involved in the operation of a specific descending inhibitory pathway controlling cochlear nucleus response to sound, it seems reasonable to suggest that discrimination of acoustic patterns depends on centrifugal modulation of coding processes in auditory afferent relays.

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Injection of compounds containing 'energy-rich' phosphate bonds into giant nerve fibres.

By P. C. Caldwell, A. L. Hodgkin and T. I. Shaw. *Laboratory of the Marine Biological Association, Plymouth*

Caldwell & Keynes (1957) showed that injection of ATP or arginine phosphate into squid giant axons which had been poisoned with cyanide caused a transient restoration of the sodium efflux. The Na efflux resulting from an injection of ATP was less than that in an unpoisoned fibre and it differed from the normal efflux in being insensitive to the external potassium concentration (see Keynes, 1958). The inference is that although an injection of ATP has some activating action on sodium transport, it does not restore the normal coupling between Na and K movements.

In further tests, with a slightly improved method, we have examined the effect of injecting five compounds containing 'energy-rich' phosphate bonds, namely, ATP, ADP, arginine phosphate, phosphoenolpyruvate (two experiments) and creatine phosphate (one experiment). The first four increased the Na efflux of a fibre poisoned with cyanide, but creatine phosphate, on which arginine phosphoryltransferase has no action (Ennor & Morrison, 1958), was without effect. ATP and ADP did not restore K sensitivity, but high concentrations of arginine phosphate ($>$ 10 mM in the axon) or phosphoenolpyruvate produced a sodium efflux which was reduced by removing external K.

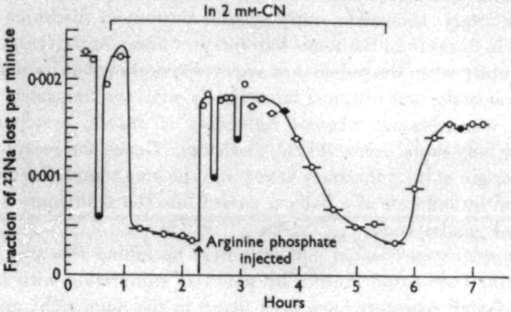

Fig. 1. Effects of injecting arginine phosphate on Na efflux of fibre poisoned with cyanide. Abscissa: time. Ordinate: ^{22}Na collected per minute per ^{22}Na in fibre; ○, with 10 mM-K in external fluid; ●, with K-free external fluid. The ^{22}Na was injected over 1 cm and the arginine phosphate over 1·5 cm with 0·25 cm overlap at both ends. 120×10^{-9} mole arginine phosphate per centimetre. Axon diameter 686 μ. Temperature 18° C.

With a high concentration of arginine phosphate, the sodium efflux of a poisoned fibre rose rapidly to a saturating level which was intermediate between the effluxes in a cyanide-free medium at the beginning and end of the experiment; this efflux was initially reduced to about half when external K was removed (Fig. 1).

These results provide further support for the view that metabolism drives the Na–K transport system by generating 'energy-rich' phosphate bonds, and draw attention to the possible role of substances like arginine phosphate and phosphoenolpyruvate.

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Receptors in the pulmonary artery.

By J. C. G. COLERIDGE and C. KIDD. *Department of Physiology, University of Leeds*

Histological evidence of receptors in the pulmonary artery of mammals has been provided by several workers (e.g. Larsell & Dow, 1933). However, apart from the recording of impulses from a single receptor located in the pulmonary artery in a cat (Swan & Whitteridge, 1956), demonstration of the existence of such receptors using electro-physiological techniques is lacking. Therefore a further attempt has been made to record the impulse activity in vagal fibres whose endings could be located with certainty in the pulmonary artery.

Afferent impulses have been recorded from 'single fibre' preparations dissected from slips of the cervical vagus in anaesthetized dogs. Recordings, made from several fibres whose endings were subsequently located in the pulmonary artery, showed an early systolic pattern of discharge similar to that found in fibres from the aortic baroreceptor area. Activity in these fibres ceased abruptly when the pulmonary artery was occluded at its junction with the right ventricle, and returned immediately with the first heart beat after the vessel was released; whereas tightening of snares, previously placed around the lung roots, increased the discharge. Hence the receptors lay between the origin of the pulmonary artery and the lung roots. Further location was effected by inflation of a balloon passed into the pulmonary artery from the external jugular vein.

The receptors were located more precisely by killing the animal rapidly and exploring the opened arteries by punctate stimulation with a fine glass probe. So far all receptors have been found in the main right and left pulmonary arteries proximal to the origins of the lobar branches. No receptors have been found in the main pulmonary arterial trunk.

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Chlorpromazine inhibition of reactions of rats to unfamiliar surroundings.

By HANNAH STEINBERG and R. H. J. WATSON. *Department of Pharmacology and M.R.C. Group for the Experimental Investigation of Behaviour, University College London*

If rats which have been reared under uniform conditions are suddenly moved to an unfamiliar environment, they usually eat and drink less for the first few days and lose weight (Steinberg & Watson, 1959). The effects of small daily doses of chlorpromazine on these reactions have been studied.

Male rats lived three per cage and were little handled until they were 200–210 days old. They were then divided into three groups. Group 1 stayed in their home cages in the colony room and were merely weighed daily. Group 2 received orally 2 mg/kg chlorpromazine hydrochloride, and Group 3 a water control. Groups 2 and 3 were moved to another room where the rats were separated from their cage mates and were installed singly in fresh cages. Food (diet 41 *b*) and water remained available *ad lib.*, but powdered food was substituted for cubes. For 3 weeks similar doses of chlorpromazine or of water were given daily and the animals were weighed. The daily consumption of food and water was recorded.

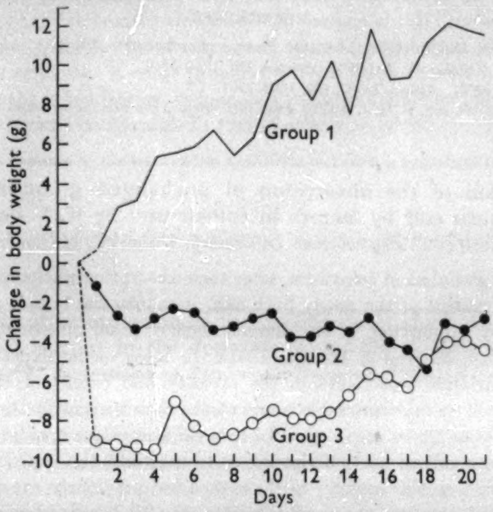

Fig. 1. Chlorpromazine inhibition of weight loss induced by removing rats to unfamiliar surroundings. Group 1 (10 rats) remained in their usual surroundings throughout. Groups 2 (7 rats) and 3 (12 rats) were moved to an unfamiliar environment at the beginning of the experiment; Group 2 received 2 mg/kg chlorpromazine daily, Group 3 a water control. Weight changes are expressed as mean gains or losses from the mean body weight of each group at the beginning of the experiment.

Twenty-four hours after the move the controls had eaten and drunk less than animals treated with chlorpromazine, and their mean body weight had dropped by 9·0 g, compared with 1·1 g for treated rats ($P < 0{\cdot}01$). After some days the controls were eating and drinking as much as treated rats and they began to recover weight. In treated rats the food and water consumption after the first day was stable and their body weight changed relatively little throughout the three weeks. However, the rats which had remained in the colony room gained 11·8 g during this period (Fig. 1).

When similar daily doses of chlorpromazine or of control were given to rats which were used to their surroundings and to being handled, the drug had no beneficial effect on body weight, but somewhat decreased it.

The results therefore suggest that chlorpromazine partly protects rats against the adverse effects which removal to strange surroundings can have on eating, drinking and on body weight. This is consistent with actions of this drug on other kinds of response to changes in the environment (Lasagna & McCann, 1957; Burn & Hobbs, 1958; Ryall, 1958).

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Acceleration of the absorption of unchanged globulins in the new-born calf by factors in colostrum.

By W. E. BALFOUR and R. S. COMLINE. *Physiological Laboratory, University of Cambridge*

Most of the globulins of colostrum, after their absorption unchanged through the small intestine of the newly born calf, pass into the lymph and can be recovered by cannulation of the thoracic duct or main intestinal lymphatic duct (Comline, Roberts & Titchen, 1951). In these earlier experiments considerable variation was found in the rapidity and extent of the globulin absorption: in the present series some of the constituents in the colostrum which may possibly be responsible for this variation have been identified.

Male Jersey calves, up to 17 hr old, were used and the experiments were conducted in a similar manner to those reported previously except that the protein introduced into the small intestine was ^{131}I labelled bovine serum γ-globulin (Armour). Its appearance and concentration in the lymph were measured with a flow Geiger–Muller tube and scaler. The influence of various substances on the globulin absorption was assessed by comparison of the times of its first appearance and the development of its maximum concentration in the lymph.

A 2% solution of this γ-globulin in whey, from which the heat coagulable protein had been removed, was absorbed at comparable rates and in similar amount to colostrum globulins introduced into the duodenum as fat-free colostrum whey. In both cases the maximum concentration of the globulin detected in the lymph was 0·4–0·6 g/100 ml. which was reached 60–120 min after its first introduction into the duodenum.

When γ-globulin (2 g/100 ml.), dissolved in a chloride solution of the same cation composition as whey, was introduced the concentration reached in the lymph rose to only 0·04–0·06 g/100 ml. even after 300 min. Replacement of some of the chloride by inorganic phosphate increased the amount of γ-globulin in the lymph; the effect was enhanced by further addition of glucose-6-phosphate, an important fraction of the bound phosphate of colostrum whey. With all these forms of phosphate there was a long delay before the appearance of the maximum concentration of globulin in the lymph. A substance whose principal effect would be to reduce the delay in the absorption has been sought.

Evidence on the nature of such a substance present in heat-treated colostrum whey has been obtained from a study of the acid resistance of the accelerating factor present in the heat-treated whey, the passage of this substance through very porous celloidin membranes and from fractionation studies with ammonium sulphate. A small protein has been prepared which, when combined with the other factors, reduces the delay to a large extent so that the absorption is more comparable to that of colostrum whey.

Part of the expenses at the start of this work were met from a grant from the Agricultural Research Council.

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The effects of stimulation of the carotid body chemoreceptors on cardiac output in the dog.

By M. de Burgh Daly* and Mary J. Scott*. *Department of Physiology, University College London*

Dogs were anaesthetized with a mixture of chloralose and urethane preceded by morphine hydrochloride. The carotid body chemoreceptors, isolated from the circulation, were stimulated by changing the perfusate from oxygenated to hypoxic blood obtained from a donor dog (Daly & Scott, 1958). Cardiac output was measured by the dye-dilution method. In some experiments in which the animals were artificially ventilated, changes in cardiac output were inferred from continuous measurements of pulmonary lobar blood flow using a rotameter flowmeter (Daly, 1957).

It was found, in animals with open or closed chest artificially ventilated by means of a Starling 'Ideal' pump, that stimulation of the carotid bodies by hypoxic blood invariably caused bradycardia, a decrease in cardiac output and an increase in calculated total peripheral vascular resistance. No appreciable changes in systemic blood pressure occurred. On the other hand, in dogs spontaneously breathing room air, chemoreceptor stimulation caused an increase in respiratory minute volume and a fall in systemic blood pressure. The cardiac output usually increased but in a few experiments it either decreased or remained unchanged. The total peripheral vascular resistance, however, invariably decreased.

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These findings suggest that the cardiovascular effects observed in the spontaneously breathing animal in response to chemoreceptor stimulation might be secondary to the concomitant reflex stimulation of respiration either by initiating a reflex from the lungs (Daly & Scott, 1958) and/or by lowering the arterial blood pCO_2 (Burnum, Hickam & McIntosh, 1954). Experiments designed to test this hypothesis showed that, in spontaneously breathing dogs, the increase in cardiac output occurring on chemoreceptor stimulation was converted to a decrease by combined denervation of the lungs and maintenance of a constant arterial blood pCO_2 by administration of a gas mixture containing 7–10% CO_2 and 21% O_2 in N_2; the increase in heart rate and decrease in total peripheral vascular resistance were also reversed. Either of these procedures alone modified the cardiovascular response to chemoreceptor stimulation, but did not always reverse them.

These results indicate that the primary cardiovascular reflex effects of chemoreceptor stimulation are bradycardia, a decrease in cardiac output and an increase in total peripheral vascular resistance, but that these effects are partly or wholly masked in the spontaneously breathing dog as a result of the concomitant stimulation of respiration.

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The effect on rate of heat production of grouping new-born pigs together.

By L. E. MOUNT. *A.R.C. Institute of Animal Physiology, Babraham, Cambridge*

New-born pigs huddle together in a cold environment, and separate from each other when the surroundings become warmer. Observations have been made to determine any influence which this behaviour may exert on the marked increase in heat production shown by the animal when the environment cools (Mount, 1958*a*, *b*).

The total oxygen consumption rate of groups of four to six pigs together, during the first week of life, was measured in a closed circuit system over the temperature range 10–36° C. Oxygen consumption rate per unit body weight increased as temperature fell, but the absolute values at any given temperature were significantly smaller when measured on the group, as compared with measurements on a single pig, at all temperatures below 30° C; the lower the temperature, the greater was the proportionate effect. At 20° C, the rate for the group was approximately one-third less than the rate for single pigs.

When huddled together, the group of pigs behaves effectively like a single larger pig in respect of the decreased heat production rate per unit body weight. Rectal temperature is maintained at the same or higher levels than those found for the single new-born pig with its higher rate of energy expenditure.

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Possible independence of oxytocin and vasopressin secretion in the human being. By S. E. Dicker. *Department of Pharmacology, University College London*

Theobald (1958) in a communication to the Physiological Society suggested that the secretion of oxytocin in the human being might be independent of that of vasopressin. This hypothesis has been tested in women during the first stage of labour and/or two days after parturition. Contractions of the uterus were recorded by means of an external tocograph. To stop neurohypophysial secretions, patients received 1000 ml. H_2O or 30–50 ml. ethanol in 200 ml. H_2O to drink. Contractions of the uterus appeared to be little affected during the ensuing diuresis. In an attempt to see whether nicotine would stimulate the secretion of both oxytocin and vasopressin, women were asked to smoke 2–3 cigarettes during the peak of a water diuresis. A marked antidiuretic effect equivalent to the intravenous infusion of 1–2 mu. vasopressin/min was observed, together with a decrease of the uterine contractions. This, however, might have been due to a release of adrenaline by the nicotine. The results of the present experiments suggest that oxytocin is not necessarily released together with vasopressin in human beings.

The author wishes to thank Professor W. C. W. Nixon for allowing this investigation to be carried out in University College Obstetric Hospital.

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The blood-brain barrier. By H. Davson and E. Spaziani*. *Medical Research Council Department of Physiology, University College London*

The existence of a blood-brain barrier has been demonstrated by measuring the rates of penetration of ^{24}Na, ^{131}I, CNS, etc. from plasma into the brain tissue; the half-life for equilibration in brain is measured in hours compared with only seconds for the extracellular space of muscle. However, on the basis of electron microscopical evidence, it has been argued (e.g. by Wyckoff & Young, 1956, and Maynard, Schultz & Pease, 1957) that the relative impenetrability of the brain to many substances is due to the virtual absence of an extracellular space in this tissue, so that equilibration with plasma is essentially a matter of penetration of cells and not the consequence of any special feature of the capillary endothelium and other layers interposed between blood and nervous tissue. If this is true, we may expect the uptake of ^{131}I by brain *in vitro* to show a much smaller effective diffusion coefficient than that observed in muscle.

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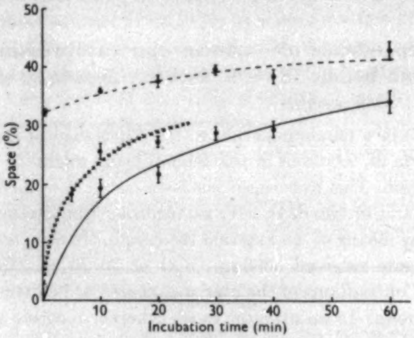

Fig. 1. Penetration of ^{131}I into brain (full curve) and diaphragm (crosses) together with changes in brain chloride space (dashes). Ordinate: percentage of tissue that would be occupied by a fluid with same concentration of ^{131}I as in medium.

Pieces of otherwise intact rabbit cerebral hemisphere were incubated at 40° C under O_2 for periods up to 60 min in Krebs-phosphate medium containing ^{131}I; the uptake of this isotope, and any changes in chloride space, were measured and plotted (Fig. 1) together with the uptake of ^{131}I by pieces of diaphragm. When allowance is made for the smaller thickness of the diaphragm, the rates of equilibration are comparable, giving effective diffusion coefficients of 5·2 and $3{\cdot}5 \times 10^{-6}$ cm^2/sec for brain and muscle respectively, compared with $1{\cdot}8 \times 10^{-5}$ cm^2/sec for free diffusion in H_2O. This contrasts with experiments *in vivo* in which, after 2 hr of intravenous infusion of ^{131}I, the brain is only some 3% of the way towards equilibrium with the blood, whilst muscle reaches its equilibrium within the shortest time studied (6 min). These results strongly support the concept of a barrier to diffusion from blood to nervous tissue.

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The activation of cholinergic vasodilator nerves in the human forearm during emotional stress.

By D. A. BLAIR, W. E. GLOVER, A. D. M. GREENFIELD and I. C. RODDIE. *Department of Physiology, The Queen's University of Belfast*

Normal subjects were either frightened, worried or embarrassed by means judged likely to be most effective for each individual. Within seconds of commencing the emotional stress there was a large increase in forearm blood flow, sometimes to as much as 40 ml./100 ml./min. There was a considerable variation in the response of different individuals to the same stressing stimulus, but in each individual the size of the response was related to the degree of emotional disturbance which he subsequently admitted. The flow rapidly subsided to normal when the subjects were reassured, comforted and consoled. Certain subjects were asked to worry themselves by thinking of unpleasant things and in some this led to an increase in forearm blood flow. During stress calf blood flow paralleled forearm blood flow, but hand blood flow was usually little changed and was never greatly increased.

The increase in forearm flow during stress was reduced and occasionally abolished either by blocking the radial, median and ulnar nerves to the forearm, or by infusion of 0·2–0·4 mg atropine sulphate into the brachial artery.

It was concluded that cholinergic vasodilator fibres to forearm blood vessels discharge during emotional stress. Evidence has been found for vasodilator fibres to forearm skin (Grant & Holling, 1938; Edholm, Fox & Macpherson, 1957; Roddie, Shepherd & Whelan, 1957) and to forearm muscle (Barcroft & Edholm, 1945). There is abundant evidence for the existence of cholinergic vasodilator fibres to skeletal muscle in animals (Uvnas, 1954) and Abrahams & Hilton (1957) have shown that these fibres are discharged by stimuli which provoke the flight and fight reactions in conscious cats. It is very probable that cholinergic vasodilator nerves to muscle are responsible for the nervous component of the changes in blood flow now reported, but a contribution from skin vasodilator nerves has not been excluded.

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Irin-like activity of some further unsaturated hydroxy-fatty acids.

By N. Ambache and Margaret Reynolds. *M.R.C. Ophthalmological Research Unit, Institute of Ophthalmology, Judd Street, London, W.C.* 1

The structural requirements for irin-like activity appear to be an OH group and a double bond in a fatty acid molecule. In view of the spasmogenic properties of (+)-ricinoleic and (+)-*iso*-ricinoleic acids already reported, the following hydroxy-fatty acids (HFA), in neutral or weakly alkaline solutions or suspensions, have now been tested on atropinized hamster colons.

Acetylenic acids

(1) (±)-*Ricinstearolic* (12-*hydroxy-octadec*-9-*ynoic*) had an activity comparable to that of ricinoleic, of which it is the acetylenic analogue (*B* in Fig. 1).

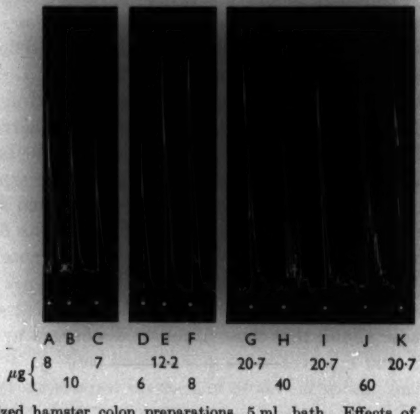

Fig. 1. Atropinized hamster colon preparations, 5 ml. bath. Effects of ricinstearolic (A, C), ricinelaidic (D, F) and *cis*-8-OH-ximenynic (H, J) acids, compared with ricinoleic (B, E, G, I, K).

(2) (±)-12-*hydroxy-dodec*-10-*ynoic* was inactive (20–100 µg/5 ml. bath).

(3) (±)-6-*hydroxy-dodec*-7-*ynoic*, inactive (50–300 µg) but depressed irin-contractions.

Thus an acetylenic unsaturated group fails to confer activity when adjacent to the OH group of HFA, but can do so when it is removed by one CH_2 as in ricinstearolic.

Ethylenic or mixed acids

(4) (+) *Ricinelaidic* (12-*hydroxy-trans-octadec*-9-*enoic*). Following Mangold's (1894) method for converting liquid (+)-ricinoleic into its *trans* isomer a white solid sample of ricinelaidic acid was obtained (m.p. 49–49·4° C after several

recrystallizations. C: found, 72·8%; calculated, for $C_{18}H_{34}O_3$, 72·43%. H: found, 11·6%; calculated, 11·48%). A 2×10^{-5} (w/v) solution of this was one to two times as active as (+)-ricinoleic (D–F).

(5), (6) *α and β kamlolenic* (18-*hydroxy-octadec*-9,11,13-*trienoic*) *acids*. Both the all-*trans* β and the α(*cis* 9) acids dissolved incompletely at 10^{-4} dilution and were then inactive in 0·5–1 ml. doses.

(7) (±)-*cis*-8-*hydroxy-ximenynic* (8-*OH-octadec*-9*yn*-*cis*-11-*enoic*) in 4–50 μg doses elicited contractions followed, after washing, by some depression of irin-contractions (G–K). Activity in relation to (+)-ricinoleic was half at low, and one-third to one-quarter at higher, dose levels, suggesting self-block.

(8) (±)-*trans*-8-*hydroxy-ximenynic*. An unpurified sample (> 80%) of this *trans*-isomer failed to contract (10–200 μg) and subsequently depressed irin.

(9), (10) *Aromatic hydroxy-acids*. *p*-Hydroxybenzoic (100–400 μg) and 3,4-dihydroxybenzoic acids (20–200 μg) were inactive; 200 μg of the latter depressed irin contractions.

We are indebted to Dr L. Crombie and Mr B. P. Griffin for the synthetic compounds 1–3 and 5–8, and to Mr S. Bance (May and Baker Ltd, Dagenham) for the micro-analysis of the ricinelaidic.

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The effect of pressure on the rate of formation of the aqueous humour.

By M. E. Langham. *M.R.C. Ophthalmological Research Unit, Institute of Ophthalmology, Judd Street, London, W.C.* 1

In a previous communication (Langham, 1958) evidence was presented that an increase in the intra-ocular pressure causes a decrease in either the rate of formation or the resistance to outflow of the aqueous humour. Continuing this investigation a method has been developed for isolating the process of aqueous humour formation, and preliminary results of the effect of pressure on aqueous humour formation are given. The outflow system was blocked by replacement of the aqueous humour of the anterior chamber by liquid paraffin; a hypodermic needle, connected to a pressure manometer and a horizontal micro-burette, was then introduced into the posterior chamber. Figure 1 is a record of the rise in intra-ocular pressure when the outflow vessels of a cat were blocked in this manner. It shows how aqueous humour formation continued until the pressure approached the diastolic blood pressure. On the assumption that the change in pressure reflected the rate of formation of the aqueous humour, and using published data of the pressure–volume relations in cats' eyes (Macri, Wanko, Grimes & von Sallmann, 1957) calculations were made of the rate of aqueous humour formation at different pressures. These indicated that aqueous humour formation decreased as the intra-ocular pressure increased. This was confirmed using the micro-burette to record formation rates at constant pressures.

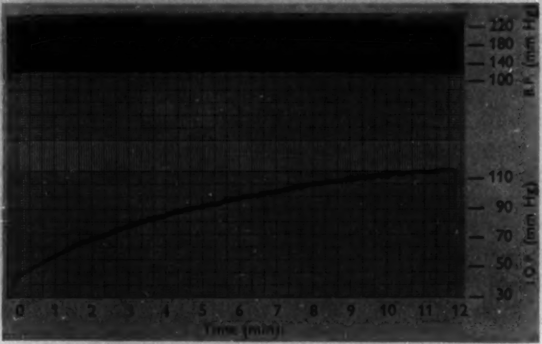

Fig. 1. Manometric record of the femoral arterial blood pressure and the intra-ocular pressure (I.O.P.) after blockage of the outflow vessels with liquid paraffin. The cat (wt. 3·4 kg) was anaesthetized with urethane.

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Peripheral resistance in the heart-lung preparation. By I. A. Boyd.

Institute of Physiology, University of Glasgow, Glasgow, W. 2

Passive transfer of autosensitization by sperm. By J. Baum, J. L. Mongar and H. O. Schild.

Department of Pharmacology, University College London

Autosensitization by sperm was first described by Metalnikoff (1900) who showed that the serum of guinea-pigs injected with their own sperm immobilized their sperm. More recently it was shown that guinea-pigs injected with sperm or testis + Freund's adjuvant exhibit testicular damage and aspermatogenesis (Freund, Murray & Thompson, 1953) and give anaphylactic reactions when treated with sperm (Freund, Thompson & Murray, 1955; Katsh, 1958). Conclusive evidence that this is a true anaphylactic sensitization has, however, been lacking, since it has not been shown so far that the sensitization can be passively transferred by serum containing circulating antibody. We have therefore attempted a passive transfer of the sensitization by methods previously described (Mongar & Schild, 1957).

In confirmation of the earlier workers it was found that male or female guinea-pigs injected with sperm or testis + Freund's adjuvant developed antibodies which immobilized sperm in the presence of complement (titre 1:100–1:800). The lungs of injected animals released histamine when incubated with sperm (living, or heated to 56° C or freeze-dried), proving an active sensitization. Passive sensitization could be demonstrated by incubating lung particles from normal guinea-pigs with the serum of sensitized guinea-pigs; when these lungs were subsequently treated with sperm they released histamine (over 10 times the control value). Passive sensitization could also be achieved with serum which had been heated to 56° C. Thus in this sensitization with homologous sperm antibodies are produced which immobilize sperm in the presence of serum complement and which become fixed on tissues and give typical anaphylactic reactions in the absence of serum complement. It is not known whether the same antibodies are responsible for the two reactions.

This work was done during the tenure by one of us (J. B.) of a Clinical Trainee-ship from the National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland, U.S.A., held at the Rheumatism Research Unit, Taplow.

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The effect of reserpine on the response of the rabbit ileum and colon to stimulation of their extrinsic nerves *in vitro*. By J. S. GILLESPIE and B. R. MACKENNA. *Department of Physiology, University of Glasgow*

The effect of reserpine (Serpasil) on the response of the intestine to stimulation of the extrinsic sympathetic and parasympathetic fibres has been studied. Two types of *in vitro* preparations were used. First, lengths of colon in which both sympathetic and parasympathetic (pelvic) nerves were retained and could be separately stimulated (Garry & Gillespie, 1955) and secondly lengths of ileum plus mesentery and mesenteric vessels; the mesenteric artery was drawn into a fluid electrode and the periarterial nerves stimulated. The rabbits were injected with reserpine intravenously for 5 days. 0·2 mg/kg for the first 3 days and 1 mg/kg for the last 2 days. In some preparations instead of pre-treating the animals for 5 days in this way the reserpine was added to the bath fluid; in these conditions its blocking action takes some hours to develop.

The inhibitor response of the colon to stimulation of the sympathetic nerves and that of the ileum to stimulation of the periarterial nerves is converted to a motor response by reserpine whether given intravenously before killing the animal, or added to the bath during the experiment. The motor response of the colon to stimulation of the parasympathetic nerves as well as the motor response of the smooth muscle to acetylcholine, and the inhibitor response to noradrenaline and adrenaline are unaltered.

The motor response to stimulation of the sympathetic nerves in both preparations is abolished by atropine and hexamethonium in concentrations identical to those required to abolish the motor response of the colon to stimulation of the parasympathetic nerves. The sensitivity of this sympathetic motor response to varying frequencies of stimulation after reserpine is similar to that of the parasympathetic nerves and unlike the sympathetic inhibitor response which it replaces.

If either colon or ileum is exposed to adrenaline or noradrenaline in fairly high concentration (10^{-5} of the base) for some time (15 min or more) then on washing the catechol amines away and re-stimulating the sympathetic nerves the original inhibitory response is temporarily restored. Repeated periods of stimulation, or one long period of stimulation, readily exhaust the inhibitory effect of nerve stimulation.

The likeliest explanation of these results is that reserpine discharges the catechol amines in the sympathetic nerve endings and so abolishes their inhibitory effect. The motor response which is uncovered is apparently due to preganglionic cholinergic fibres, almost certainly vagal in the preparations of ileum, and probably pelvic in origin in the colon preparation. The restoration of inhibition after soaking the preparations in noradrenaline or adrenaline indicates a re-incorporation of these amines in the nerve endings, similar to that demonstrated by Burn & Rand (1958).

The Rankin Medical Research Fund of the University of Glasgow helped to supply apparatus.

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PROCEEDINGS OF THE PHYSIOLOGICAL SOCIETY

PHYSIOLOGY INSTITUTE, NEWPORT ROAD, CARDIFF

17–18 *April* 1959

An electronically controlled constant-velocity microtome. By

G. B. David and R. C. Steel. *M.R.C. Neuropsychiatric Research Unit, Whitchurch Hospital, Cardiff*

Special equipment was developed in order: (i) to obtain routinely series of thin sections of nervous tissue, cut at a constant velocity; (ii) to study the relation between velocity of cutting and distortion of the finished sections. The equipment is illustrated in Fig. 1.

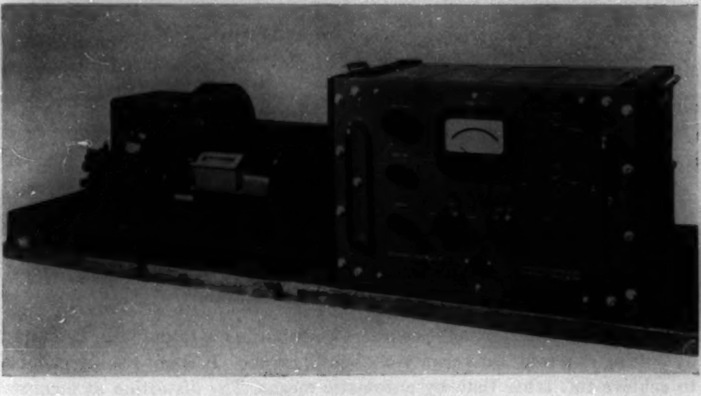

Fig. 1. The complete microtome.

The mechanical parts are assembled on a cast-iron base, 60 × 60 × 2·5 cm. A Spencer No. 820 [American Optical Co., Buffalo, U.S.A.] microtome is driven by a d.c. motor-generator, the output of which is reduced by a 30:1 worm-and-nut gearbox. An electro-magnetic switching counter is also attached to the motor-generator. The d.c. motor-generator has a rating of $\frac{1}{8}$ h.p. at 6000 r.p.m., and is controlled by a modified Servomex [Jarvis Brook, Sussex] No. 43 motor controller, housed in a separate case.

In operation, the motor is run at a constant current of 5 A. The generator, driven by the motor, has a d.c. output linearly proportional to the velocity of the shaft. A fraction of this voltage is compared with a pre-set reference voltage; the difference between these is amplified, and used to drive the paired output valves that control the field current of the motor. This feed-back arrangement results in constant torque being available throughout the useful range of cutting velocities. The output of the generator is also displayed on a meter, calibrated in r.p.m. The torque available at the microtome is 4×10^4 g/cm. The cutting velocity can be continuously varied between 0·17 and 33·0 cm/sec. Errors in angular velocity are corrected at a rate of 9000 r.p.m./sec; in practice, the cutting velocity was found to be constant within 0·1 % of the nominal setting.

Two measures of distortion were found to be correlated with cutting velocity: reduction of surface area through compression, and increase in thickness through expansion in the remaining dimension; the former was estimated planimetrically, and the latter interferometrically. The results of measurements on fifty complete frontal sections of the brain of the cat, cut at 7 μ, and at different velocities, are given in Table 1.

Table 1. The effect of varying the cutting velocity on the distortion of 7 μ paraffin sections of the cat brain: percentage reduction in surface area* and percentage increase in thickness:† means of measurements on ten sections cut at each velocity

Velocity (cm/sec)	Reduction in surface area, %,* ±S.E.M.		Increase in thickness, %,† ±S.E.M.	
2	8·8	±1·07	3·8	±1·33
4	9·1		5·0	
6	13·9		8·8	
8	16·6		13·2	
10	19·2		19·0	
Mean	13·5 ± 0·48		10·0 ± 0·6	

$$* \; 100 \times \frac{\text{surface area of the block} - \text{surface area of the section}}{\text{surface area of the block}}.$$

$$\dagger \; 100 \times \frac{\text{nominal thickness of the section} - \text{measured thickness of the section}}{\text{nominal thickness of the section}}.$$

The amount of distortion encountered in routine microtomy can be halved by cutting at a constant velocity of about 2 cm/sec. It is not readily possible to achieve this other than by automatic means. The distortion at 2 cm/sec can be reduced to less than 2 % if the knives used with the special microtome are true and plane to within ± 0·1 μ.

A temperature-controlled watch for use in acclimatization studies in the field.

By H. S. Wolff. *Bioengineering Laboratory, Division of Human Physiology, National Institute for Medical Research, London, N.W.* 3

Much of recent expedition physiology has centred around the influence of climate on the human subject, and the investigations have often taken the form of attempting to detect signs of acclimatization. There has always been the difficulty, however, that man, being an ingenious animal, protects himself against climatic stress either by living in a conditioned atmosphere or by wearing appropriate clothes. A simple instrument was therefore needed to assess how much time the subject spends outdoors, or to assess for how long the temperature of some specified layer of his clothing is outside temperate limits. Only when this information becomes available is it possible to correlate physiological differences, or the lack of them, with exposure.

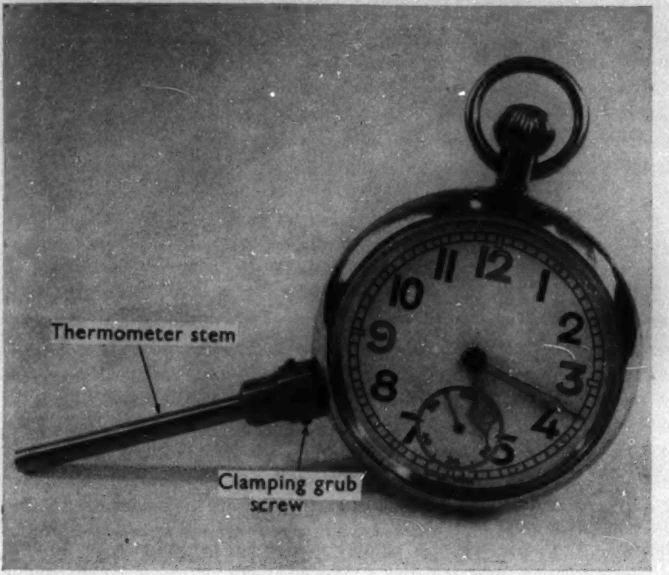

Fig. 1

The device (Fig. 1) is an ordinary pocket watch which has a plastic-lined bush attached to one edge of the case close to the balance wheel. The stem of a bimetallic thermometer fits into the bush so that its output shaft, which normally carries the pointer, projects into the watch (Fig. 2). The original pointer (*P*), suitably bent, is re-attached to the shaft so that it can interfere with the adjusting screws on the balance to stop the watch. There is enough room in the watch case for the pointer to swing clear of the balance. By turning the stem of the thermometer in the bush the watch can be set to stop when the temperature falls below a pre-set level. By a slightly different internal arrangement, it can also be made to stop when the temperature rises above a pre-set point. If the problem is to discover how much time is spent at temperatures below, say, −20° C the watch is adjusted to stop at −20° C. It is set to the correct time every morning, attached to the subject and read the following morning. The time it has lost will be equal to the time spent below −20° C. The use of a number of watches per subject, set to stop at different temperatures, will give a more complete picture of his environment. The device is cheap and can be used to follow the exposure pattern of groups of people.

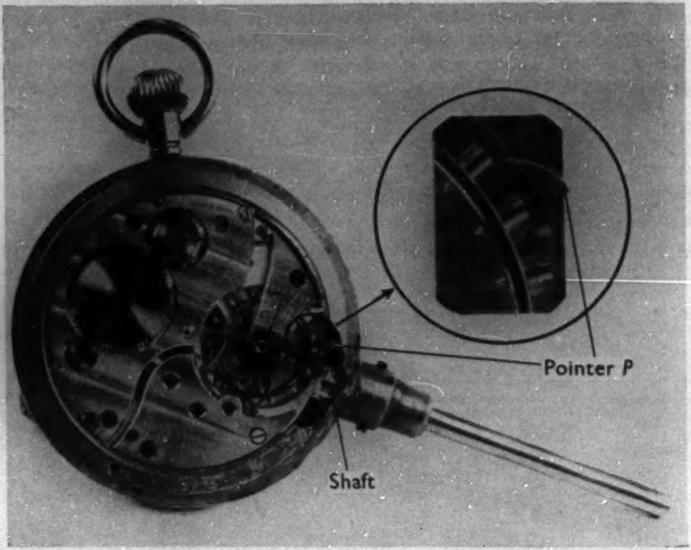

Fig. 2

Mitotic activity in mesenchymal cell types of the guinea-pig gall-bladder following ligation of the common bile duct. By F. Jacoby. *Sub-Department of Histology, University College, Cardiff*

The high mitotic activity occurring in the epithelium of the distended guinea-pig gall-bladder following ligation of the common bile duct and also its time course have previously been demonstrated (Jacoby, 1953). After a latent period of about 18 hr proliferation begins and reaches a peak as early as 24 hr after operation. Mitotic activity remains high for two further days, drops sharply on the fourth and then comes down to a somewhat subcontrol level on the 6th day.

Further analysis of the sectioned material of these gall-bladders has revealed that besides the epithelium all other cell types that are capable of division are also stimulated to mitotic proliferation, but with time courses that differ from that of the epithelium and are generally on a lower scale. They all lag, by about a day, behind the mitotic activity of the epithelium; that is to say they only begin to show mitosis at a time when the epithelial activity has already reached its peak. Serosal endothelium, connective tissue cells and vascular endothelium show their highest proliferation rate between 48 and 72 hr after operation and then gradually return to rest. In contrast, smooth muscle cells of the muscular coat of the gall-bladder, though starting after the same latent period, continue to multiply mitotically at a climbing rate up to the 5th or even 6th day, when these experiments were terminated.

The demonstration consists of histological specimens to show the mitotic activity of all these cell types at different times after operation. Some mitoses have been found even amongst the muscle cells of the tunica media of arteries; in a single instance a cell in mitosis was seen in the lumen of a vessel (? dividing macrophage).

The observations indicate the presence of a generalized mitotic stimulus passing over the organ and are consistent with the idea of a functional hyperplasia. The two main operative tissues of the gall-bladder are (*a*) the epithelium concentrating the bile, and (*b*) the muscle coat periodically emptying the organ. After ligation of the common bile duct with its consequential great influx of bile into the gall-bladder, the epithelium is the first to respond, by an increase of its cell population, to the increased functional demand. The muscle coat follows only gradually; its functional hyperplasia occurs in response to the ever-increasing resistance due to the tied bile duct; hence mitoses in this tissue are still frequent 6 days after the operation. In addition, there is a hypertrophy of the muscle cells. Hyperaemia is evident throughout the organ, and the mitotic activity of some of the other cell types may be merely incidental under conditions generally favourable for mitosis, though the proliferation of the vascular endothelium may have been evoked by the enlargement of the vascular bed. The only cell type whose proliferation may, in part, be due to *regeneration* is the serosal endothelium, as slight damage to the gall-bladder surface, at operation, is practically unavoidable.

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Observations on the renal elimination of corticosteroids. By S. Shuster. *Medical Unit, Rockefeller Building, Royal Infirmary, Cardiff*

The effects of wasting on the body water. By B. McConkey. *Medical Unit, Rockefeller Building, Royal Infirmary, Cardiff*

Methods of assay of melanophore expanding hormone. By J. N. Karkun, R. A. Main and G. M. Mitchell. *Department of Pharmacology, Institute of Preventive Medicine, Cardiff*

A simple method of making a fog. By C. B. McKerrow. *Medical Research Council Pneumoconiosis Research Unit, Llandough Hospital, near Penarth, Glam*

During an investigation on the effects of changes in climatic conditions on patients with chronic respiratory disease, we needed an apparatus to produce a water-containing fog at any temperature from about 3 to 37° C.

The principle of the apparatus illustrated (Fig. 1) is to allow air, cooled to about −40° C by a mixture of solid carbon dioxide and alcohol, to come into contact with steam in an 8 l. polythene bag from which the subject breathes.

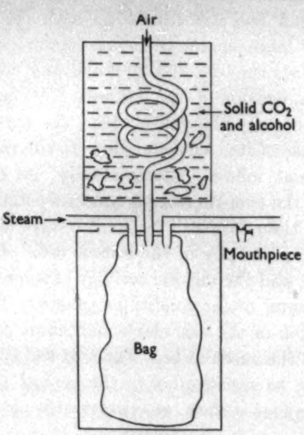

Fig. 1

The steam both provides nuclei for the fog formation and also reheats the air. Adjustment of the proportion of steam to cold air permits a wide variation in the temperature of the resulting fog.

A cuvette made from cold-setting resin.

By N. Bevan. *Medical Research Council Pneumoconiosis Research Unit, Llandough Hospital, near Penarth, Glam*

A cuvette of square cross-section has been constructed for use in the determination of cardiac output by the indicator dilution method.

When compared with a cuvette made from flattened polythene (Wood, Swan & Helmholz, 1957) this design has the advantage of eliminating the light which reaches the photocell via the wall of the tube. In addition, the lumen depth is constant and the unit can be readily mounted.

In this design (Fig. 1) the side walls of the cuvette are black Perspex; the front and back faces and ends, into which are sealed lengths of stainless-steel tube, are Ceemar cold-setting resin.*

In its construction the tubing is secured with Araldite cold-setting epoxy resin† to two pieces of black Perspex: the space between is filled with paraffin wax which is made smooth and level with the Perspex by scraping. The Perspex is then placed in a mould, through which the steel tubes protrude, and is encapsulated in the resin. The mould faces parallel to the plane of the Perspex are lined with glass to produce a plane surface with a perfect finish. When the resin is set the cuvette is heated in water and the paraffin wax expelled by blowing. Holes are then drilled through the block to facilitate mounting.

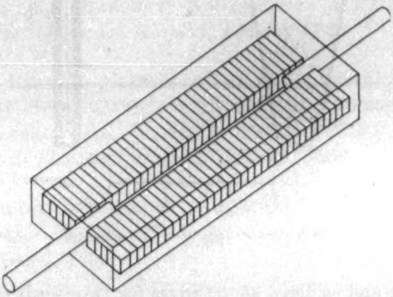

Fig. 1

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* E. M. Cromwell and Co. Ltd, Bishop's Stortford.

† Aero Research, Duxford, Cambridge.

A simple air-flow interrupter.

By A. W. Hyett and Margery McDermott. *Medical Research Council Pneumoconiosis Research Unit, Llandough Hospital, near Penarth, Glam*

The interrupter is extremely light and simple to construct. The present model is electrically operated by means of a 12V solenoid but a mechanical or hydraulic system could be used.

The interrupter (Fig. 1) consists of a sealed Perspex sandwich with an $\frac{1}{8}$ in. (3 mm) air gap in the centre. In this air gap is a sector arm (Q), to the extremity of which are araldited two mica disks $\frac{1}{100}$ in. (0·25 mm) thick. The arm operates through a fulcrum pin (F) by means of a $\frac{3}{16}$ in. movement of the control rod (C). It is made from phosphor-bronze strip $\frac{1}{4}$ in. wide and $\frac{5}{1000}$ in. (0·12 mm) thick, folded lengthwise and soldered at the edges for two-thirds of its length from the fulcrum. The remainder is left as a loop, ensuring spring sealing of the mica disks against the seats (S). These seats protrude from the inner surfaces of the sandwich by approximately $\frac{4}{1000}$ in. and they are slightly curved to ensure sliding motion of the disks. The area of contact between each disk and its seat is over an annulus $\frac{1}{64}$ in. wide.

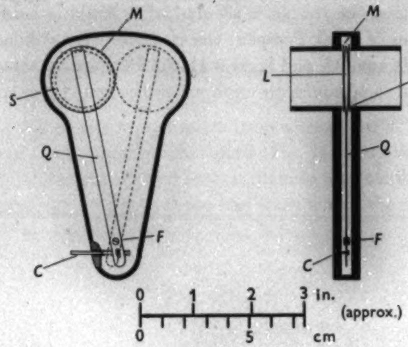

Fig. 1

The use of two disks instead of one makes a precision fit unnecessary and hence enables an interrupter to be constructed with very low power requirements. Also, during oscillating flow, if the pressure is sufficient to lift one disk from its seat, this same pressure is automatically applied to sealing the second disk. This interrupter with disks $\frac{1}{100}$ in. thick was designed primarily for respiratory flow. It can be easily adapted for use with larger pressures by increasing the disk thickness. The same principle could be used for the construction of cheap and simple multi-channel valves.

Smoking and ventilatory capacity. By I. T. T. Higgins. *M.R.C. Pneumoconiosis Research Unit, Llandough Hospital, Penarth, Glam*

Factors affecting arterial blood pressure in the general population. By W. E. Miall and P. D. Oldham. *M.R.C. Pneumoconiosis Research Unit, Llandough Hospital, Penarth, Glam*

The measurement of blood gas tensions. By J. E. Cotes. *M.R.C. Pneumoconiosis Research Unit, Llandough Hospital, Penarth, Glam*

Pulmonary diffusing capacity by the single breath method. By R. S. Jones and F. Meade. *M.R.C. Pneumoconiosis Research Unit, Llandough Hospital, Penarth, Glam*

Estimation of serum cholesterol. By T. G. Morris. *M.R.C. Pneumoconiosis Research Unit, Llandough Hospital, Penarth, Glam*

The measurement of lung volume and airway resistance. By M. Collins and Margery McDermott. *M.R.C. Pneumoconiosis Research Unit, Llandough Hospital, Penarth, Glam*

Investigations into the pharmacology and physiological effects of cotton dust. By A. Davenport, J. C. Gilson, Margery McDermott, C. B. McKerrow and W. D. M. Paton. *M.R.C. Pneumoconiosis Research Unit, Llandough Hospital, Penarth, Glam*

A nitrogen meter for the investigation of lung function. By V. Timbrell. *M.R.C. Pneumoconiosis Research Unit, Llandough Hospital, Penarth, Glam*

A double-image microscope micrometer with facilities for accurate particle-size analysis. By V. Timbrell. *M.R.C. Pneumoconiosis Research Unit, Llandough Hospital, Penarth, Glam*

Responses of the smooth muscle of *Mytilus edulis*. By G. W. Cambridge and J. A. Holgate. *Department of Physiology, School of Medicine, Leeds* 2

Previous investigations of the responses to electrical stimulation of the non-striated anterior retractor muscle of the byssus (ARMB) of *Mytilus edulis* have shown two types of response (Winton, 1936; Fletcher, 1937). The first consists of contraction followed by slow relaxation ('tonic' response) and can be evoked by d.c. at 5–10 V applied continuously for 10–20 sec through electrodes placed in contact with the muscle, or by the application of ACh in concentrations of 1–2 μg/ml. The second type of response ('phasic' response) consists of contraction followed by rapid relaxation, and this can be evoked by pulse stimulation of 300 msec width at 60/min and 5–10 V, again through electrodes placed in contact with the muscle. If this stimulus be applied during the phase of slow relaxation following a previous tonic response, the relaxation following the second contraction restores the muscle tension to resting level in a much shorter time than that following the tonic response. In such circumstances if the second stimulus be given at a frequency of 15/min, the acceleration of relaxation can be evoked with little or no immediately preceding contraction, and this can be mimicked by the application of 5-HT in concentrations of 1–10 ng/ml.

The tonic response to ACh can be blocked by ACh antagonists, the most powerful in the series tested being propantheline and the weakest dimethyl tubocurarine. Applying these antagonists to the muscle before evocation of a tonic response by d.c. stimulation does not affect the response in any way, but if a phasic response is evoked by pulse stimulation the muscle fails to contract but still shows the rapid relaxation characteristic of such a response. In this connexion the order of activity of various ACh antagonists in blocking the contractile response is the same as that found when they are used to block the tonic response to ACh. Hexamethonium is not particularly active in this respect and we have been unable to confirm the presence of ganglion cells within the muscle reported by Bowden & Lowy (1955).

It would appear that electrical stimulation through electrodes placed in contact with the muscle can bring about either (*a*) contraction by direct stimulation of the muscle elements, or (*b*) contraction through some intermediate mechanism involving ACh, or (*c*) relaxation, possibly involving the liberation of 5-HT. To some extent (*b*) and (*c*) are associated in that in the period of slow relaxation following a tonic contraction we have not yet been able to find a stimulus, which would have produced a contraction in the absence of ACh antagonists, which does not at the same time evoke acceleration of relaxation, though rapid relaxation which is not preceded by immediate contraction can be obtained.

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Mechanical factors in hyperaemia.

By J. Stubbs and W. F. Widdas.
Department of Physiology, King's College, London, W.C. 2

In experiments in which the weight of the perfused isolated rabbit heart decreased as a result of adrenaline or calcium stimulation, or increased owing to the effect of acetylcholine, there was an increase in the coronary outflow.

After the stimulation by adrenaline the loss in weight persisted longer than the increased ballistic effect and the coronary outflow remained at a higher level for a still longer period. When the heart was arrested by perfusion with Ringer–Locke solution containing potassium citrate, the outflow (initially increased) fell progressively during the period of quiescence.

By perfusing the heart with Ringer–Locke solution containing Evans Blue or a 1% suspension of washed rabbit cells and continually monitoring the outflow colorimetrically it was found that losses in weight were accompanied by a sharp reduction in coloration, confirming that extravascular fluid was lost (Stubbs & Widdas, 1958). The concentration of colour on regaining weight after a loss (e.g. that caused by adrenaline) was difficult to distinguish, as it tended to be small and prolonged. The gain in weight due to acetylcholine was not accompanied by a corresponding concentration of colour.

If the perfusion was stopped there was a reduction in weight. On restarting the perfusion there was an increased outflow and the first effluent showed a dilution. The weight was quickly regained but without a corresponding concentration of colour being evident.

The changes in the coronary flow can be correlated with weight and dye findings if the hypothesis be made that the calibre and functional volume of some resistance vessels (probably capillaries) depends, in part, on the support of muscle and interstitial fluid. It is visualized that excess loss of interstitial fluid during systole (adrenaline) or a reduced myocardial tone (acetylcholine) permits a greater capillary dilatation during diastole. A prolonged quiescence, however, by eliminating the backward filtration associated with systole, would lead to an increased interstitial fluid volume at the expense of the intravascular volume, so leading to constriction.

That peripheral resistance and circulatory capacity may be influenced by muscle tonus has been shown in the perfused intestine by Sidky & Bean (1958), but the present concept also attributes an important peripheral vascular role to changes in the interstitial fluid volume.

A grant for scientific assistance from the Medical Research Council is gratefully acknowledged by W.F.W.

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Pressure changes in the middle ear after simulated flights in a decompression chamber.

By G. Melvill Jones. *External staff M.R.C., R.A.F. Institute of Aviation Medicine, Farnborough, Hants*

After flying it is common to experience discomfort due to pressure changes in the middle ear, normally relieved by intermittently opening the pharyngotympanic tube. These pressure changes have been examined in three subjects after simulated flights in a decompression chamber during which the composition of middle ear gas was altered by breathing known gas mixtures during 'descent'. Immediately after final 'descent' and thereafter at about 5 hr intervals until approximate attainment of equilibrium, the rates of fall in middle ear pressure were determined over one hour periods of observation. Air entry through the pharyngo-tympanic tube was prohibited during, but freely permitted between, the observation periods. The apparatus employed was similar to that described by van Dishoeck (1941) which depends upon the fact that maximum loudness of a sound of constant intensity occurs at a constant pressure differential across the tympanum.

The rates of fall of pressure during each period examined were plotted against time elapsed, and the areas under these curves were found to bear a linear relation to the O_2 content of gas breathed during 'descent'. Since these areas can be shown to be directly related to the net absorption of gas across the enclosing surface it is concluded that absorption of oxygen is the primary cause of the pressure changes which occur after flight. The results indicate that O_2 continues to be absorbed until its partial pressure has fallen to approximately 65 mm Hg, which suggests that this is the normal equilibrium value in the middle ear.

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Relation between concentration and rate of intestinal absorption of D- and L-amino acids.

By E. Lesly Jervis and D. H. Smyth. *Department of Physiology, University of Sheffield*

It is generally agreed from the work of Wiseman (1953) and others that a special mechanism is involved in the transfer of L-amino acids by the intestine. Recently Jervis & Smyth (1958) have shown evidence for the participation of a special mechanism in the absorption of D-methionine. Since the relation between the rate of absorption of substances and their concentration in the intestine may throw light on the nature of the process concerned, the following experiments were undertaken to study the effects of concentration on the rate of absorption of both D- and L-amino acids.

Albino rats were anaesthetized with pentobarbitone sodium, and the method of study of absorption was that of Sheff & Smyth (1955). The D- and L-enantiomorphs of histidine and methionine were used, and determinations were made of the amounts of these absorbed in 20 min periods from initial concentrations of 2–100 mM. The results showed that for all four enantiomorphs the rate of absorption was not proportional to the concentration present in the intestine, and at higher concentrations the rate of absorption was proportionally smaller than at lower concentrations. Except in the case of L-methionine when the maximum rate was obtained at 50 mM, the absolute rate was still increasing at the highest concentration used, so that the mechanism did not appear to be saturated.

Fisher & Parsons (1953) first applied Michaelis–Menten kinetics to intestinal absorption, in their case to glucose absorption, and an attempt was made to see whether the relationship between the rate of absorption and concentration of the amino acids fitted in with Michaelis–Menten kinetics. This was done by using the Lineweaver & Burk (1934) form of the Michaelis–Menten equation, i.e. plotting the reciprocals of the rate of absorption and the concentration. This showed that for all four amino acids tested the points approximated to the straight line relationship required by Michaelis–Menten kinetics.

Previous authors, e.g. Lathe (1943), have shown that the absorption of amino acids is not proportional to their concentration, and our results are in agreement with these. They also show that the relationship between the rate of absorption and concentration is of a similar nature in the case of L- and D-enantiomorphs. This indicates that diffusion is not a rate-limiting factor in the absorption of either D- or L-enantiomorphs of histidine or methionine. The approximation to the Michaelis–Menten kinetics is consistent with the formation of a carrier-substrate complex or of an enzyme-substrate complex in the transfer mechanism. It should be stressed, however, that the existence of an enzyme-controlled process is not proved by Michaelis–Menten kinetics, as it is known that other processes may exhibit a similar kinetic relationship.

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Acetylcholine sensitivity of partially denervated frog muscle fibres.

By R. MILEDI. *Department of Biophysics, University College London*

Most muscle fibres in the frog's sartorius are innervated at two or three points along their length (Katz & Kuffler, 1941). It is of interest to find out whether denervation of only one of the end-plates in these fibres is followed by the supersensitivity to acetylcholine which is characteristic of completely denervated muscle.

Experiments were made on sartorius muscles, some of whose tibial or pelvic intramuscular nerve branches had previously been divided. This resulted in partial denervation of many muscle fibres.

A few days after the operation the miniature end-plate potentials disappeared from the denervated end-plate. Some days later spontaneous potentials at a much lower frequency reappeared, as described in a previous communication (Katz & Miledi, 1959). After a few weeks the sensitivity of the denervated end-plates to diffusely applied acetylcholine was found to be from 10 to 1000 times greater than that of the innervated end-plates, or of the normal control muscle.

In spite of the increased ACh-sensitivity the amplitudes of the miniature potentials at these 'supersensitive' end-plates were usually not much larger than those occurring at normal end-plates. This could be explained if 'supersensitivity' to the applied drug is due to an increase in the sensitive area of the fibre membrane (cf. Axelsson & Thesleff, 1959). After complete denervation, responses to electrophoretically applied acetylcholine could be obtained from almost anywhere on the muscle fibre. Nevertheless, 3 months after denervation the ACh-sensitivity at the end-plate was still greater than at other regions.

It was further found that the membrane resistance of the partially denervated muscle fibre is consistently higher in the denervated region than elsewhere. As early as 10 days after intramuscular nerve section, re-innervation of some muscle fibres has been observed. End-plates remained supersensitive for some time after transmission had been re-established.

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Changes in the lungs and chest with death.

By R. J. Shephard. *Ministry of Supply Chemical Defence Experimental Establishment, Porton Down, Salisbury, Wilts*

Although an important preliminary to a study of artificial respiration in the cadaver, little work has previously been reported on changes in the physical properties of the lungs and chest with death. The present communication compares findings in the rabbit before and up to 18 hr after respiratory failure produced by an overdose of anaesthetic (intraperitoneal pentobarbitone).

Stable pressure–volume relationships could be obtained after death if the chest was subjected to one or two slow ($\frac{1}{2}$–1 min) and deep (30–50 ml.) inflations. Within 15–30 min of death, three changes in the pressure–volume curve were observed:

(1) An exaggeration of the non-linear form.

(2) A large difference in response to the first and subsequent deflations of the lung, and

(3) A small increase in the pressure needed to inflate the lung.

All three changes became more marked in succeeding hours. (1) and (2) seemed due to collapse of parts of the lung, associated with an alteration in the quality or quantity of bronchial secretions. Both changes were slightly decreased by amyl acetate vapour. (3) normally reached a maximum 3–5 hr after death, but was hastened by a warm environment (37° C). It seemed mainly a reflexion of rigor mortis. In the open-chest preparation lesser early changes were noted, possibly owing to pooling of blood in the lungs.

In view of the complex changes in respiratory mechanics following very closely upon death considerable caution is needed in interpreting results obtained on 'fresh' cadavers.

The influence of anoxia upon the tolerance of man to pressure breathing with an oronasal mask, with trunk and lower limb counterpressure.

By J. Ernsting, I. D. Green and P. R. Wagner. *R.A.F. Institute of Aviation Medicine, Farnborough, Hants*

Anoxia at altitudes of greater than 40,000 ft. (12200 m; $P_B = 141$ mm Hg) may be prevented by positive pressure breathing with oxygen. One system which has been developed for this purpose employs an oronasal mask with counterpressure applied to the trunk and the lower limbs (Ernsting & Roxburgh, 1957). A breathing pressure of 60 mm Hg is used with this equipment at an altitude of 56,000 ft. (16760 m; $P_B = 66$ mm Hg), and this results in an alveolar oxygen tension of approximately 50 mm Hg. The influence of this degree of anoxia and the disturbances associated with the use of an oronasal mask have been investigated during pressure breathing in five subjects.

Each subject was exposed to a breathing pressure of 60 mm Hg with trunk and lower limb counterpressure for a period of 6 min on four separate occasions, twice at sea level when air was breathed and twice at a simulated altitude of 56,000 ft. when oxygen was respired. On one occasion at each altitude an oronasal mask was used. A pressure headpiece which covered the head and the upper part of the neck was worn for the other two exposures. In each experiment the electrocardiogram, the arterial blood pressure, the respiratory rate and the transparietal electro-encephalogram were recorded continuously. The performance was measured by means of a two-channel tracking task and the behaviour of the subject was recorded by means of a cine camera.

All the subjects successfully completed the two periods of pressure breathing at ground level and the exposure to 56,000 ft. when a headpiece was worn. During pressure breathing with a mask at 56,000 ft., however, two of the five subjects lost consciousness within 3 min. The circulatory changes which occurred in these two subjects were characteristic of vasovagal syncope. Since the degree of anoxia in the two series at 56,000 ft. was similar it is suggested that the combination of anoxia with the discomforts and disturbances associated with the use of an oronasal mask (Ernsting & Roxburgh, 1957) was responsible for the collapses in the final series of experiments.

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Henle's loops and the sodium content of the medullae of mammalian kidneys.

By J. A. Barclay, R. F. Crampton and D. M. Matthews. *Department of Physiology, University of Birmingham, Abbott Laboratories, London, and the Department of Pathology, Royal Free Hospital, London, W.C. 1*

Wirz, Hargitay & Kuhn (1951) proposed that the loops of Henle are the morphological elements concerned in a counter-current system which results in the concentration of tubular fluid. The sodium content of the kidney increases from the cortex to the medulla. This has been shown in the dog (Ullrich & Jaraus, 1956) and in the rat (Crampton, 1957). If the collecting tubules were impermeable to sodium, this would be expected from the counter-current hypothesis. We have therefore examined the sodium content of the medulla in several mammals, the kidneys of which differ markedly with regard to the number of loops which extend to the region of the medullary papilla. This aspect of renal morphology has been extensively studied by Sperber (1944). The animals were anaesthetized before removal of the kidneys, in order to ensure concentrated urines.

Table 1. The sodium content of the medulla of various animals compared with the percentage of long loops of Henle. Figures for sodium are means, with the number of animals given in brackets

Animal	Sodium content of medulla (m-equiv/kg wet wt.)	Long loops of Henle (%)
Cat	240 (5)	100
Dog	224 (7)	100
Rabbit	222 (6)	46–48
Rat	176 (6)	28
Pig	105 (2)	0–5

There is a marked tendency for the sodium content to vary with the number of long loops and, to this extent, the data would be as expected if a counter-current system was in operation. In the pig the sodium content was also determined in the peripheral and central portions of the medulla. The peripheral portion contains parts of all the loops whereas the central (papillary) portion contains virtually none. For two pigs the sodium content of the peripheral medulla was 79 and 89 m-equiv/kg and the corresponding values for the papillary portions were 115 and 98 m-equiv/kg.

The papillary portion of the rat medulla was also examined for sodium. Its content was found to be 177 m-equiv/kg. According to the Wirz hypothesis, the maximum concentration of sodium should occur at the apices of the loops. The results we obtained in the rat and pig are not consistent with this hypothesis.

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Changes in the cardiac output during positive radial acceleration.

By P. Howard. *R.A.F. Institute of Aviation Medicine, Farnborough, Hants*

It is known that positive radial acceleration (centrifugal force acting in the head-to-foot direction) produces profound changes in the dynamics of the circulation. The output of the heart is likely to be affected by these changes but hitherto no experimental confirmation of this assumption has been presented.

The direct Fick method was used to determine the cardiac output in two subjects. A polythene catheter was inserted into the right atrium via an antecubital vein; its position being confirmed by examination of the pressure wave form. The subject lay supine on the end of the human centrifuge, with the legs extended. Expired air (5 min collections into a Douglas bag), and mixed venous blood from the auricle were collected under the following conditions: after 10 min at rest, after the centrifuge had been accelerated to a constant speed, and after 15 min rest with the machine stationary. In one case the resultant acceleration used was 2·0 *g* and in the other it was 2·4 *g*. A sample of blood from the femoral artery was obtained immediately after the machine had come to rest. The gas and blood samples were analysed by the Haldane and Van Slyke methods, respectively, and from the results the oxygen consumption and cardiac output were calculated.

At 2 *g* the output was reduced to approximately 68% of the resting value, and at 2·4 *g* it fell to about 60%. Because of the concomitant increase in heart rate the changes in stroke volume were greater still. In both cases the oxygen consumption rose by about one-third during the exposure.

These results may be compared with those obtained after tipping from the horizontal to the erect posture (equivalent to a radial acceleration of 1 *g*) which produces an average decrease of cardiac output of 25% (McMichael & Sharpey-Schafer, 1944; Stead, Warren & Merrill, 1945).

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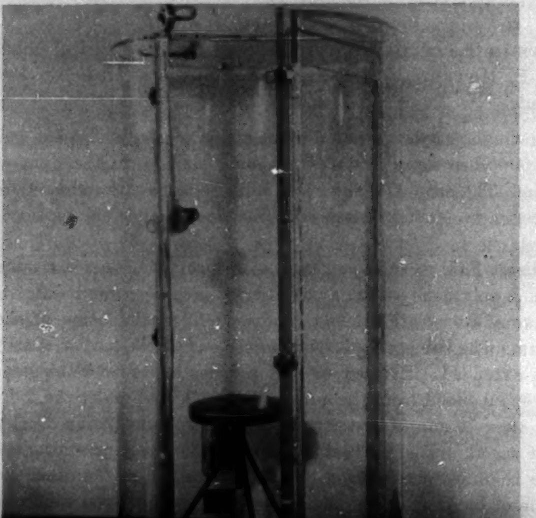
Problems in the use of a body plethysmograph. By MARGERY McDERMOTT. *Medical Research Council Pneumoconiosis Research Unit, Llandough Hospital, near Penarth, Glam*

The plethysmograph with which these investigations have been made was designed primarily for the measurement of thoracic gas volume and lung airways resistance. It was constructed from a Perspex cylinder 60 in. (152 cm) high, 29 in. diameter and ⅜ in. (1 cm) thickness, with a volume of approximately 650 l. and weight 100 lb. (45·4 kg).



It was decided to use a constant volume system in which changes in lung volume are measured as pressure changes (of the order of 0·01 cm H_2O) in the plethysmograph. This decision was made after considering the relative merits of constant volume and constant pressure plethysmographs.

Three major problems arise in the use of this type of instrument:

(1) *The stability of the pressure recorders, calibration methods, and the effect of temperature and barometric pressure variations on them.*

(2) *The stability of the temperature and pressure in the plethysmograph.*

During recording, the subject breathes against a closed shutter, or pants rapidly into the closed plethysmograph. Contrary to expectation pressure within the plethysmograph tends to decrease, although the air temperature rises. This may be due to the thermal properties of the material of the plethysmograph, the constantly rising water vapour concentration, and also the small effect of the respiratory quotient.

(3) *The phase shift between air flow and alveolar pressure recordings during the measurement of airways resistance.*

The changes of alveolar pressure during flow are calculated from measurements of the corresponding changes in the volume of air in the lungs. The observed phase shift does not vary with the volume of air moved in each breath but decreases with increasing breathing rate until it is negligible at about 1·5 c/s.

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The effect of pregnancy on inulin and para-aminohippuric acid clearances in the anaesthetized rat. By B. F. Matthews and D. W. Taylor. *Department of Physiology, University of Aberdeen*

Most methods used for determining the renal clearance values in rats have been open to criticism, but Dicker (1956) has pointed out that 'provided the urine collections are made while the concentration of inulin or para-aminohippuric acid (PAH) are changing quite slowly, the single subcutaneous injection technique may yield reasonably accurate results'.

Rats were anaesthetized with pentobarbitone, and two solutions, one containing inulin and mannitol, the other PAH, were injected at separate sites subcutaneously. Bladder urine was collected for a period of exactly 10 min from the 50th minute after injection. Within 1 min of the end of the urine-collecting period a sample of blood was obtained from the femoral vessels. The concentration of inulin in plasma and urine was determined by the method of Roe, Epstein & Goldstein (1949). PAH was determined according to the method of Bratton & Marshall (1939).

Inulin and PAH clearances were measured in two groups each of twenty-four female rats. The first group consisted of unmated animals, and the second of pregnant animals mated 18 days earlier. The main finding was a significant increase in the inulin clearance of the 18-day pregnant rats compared with that of the unmated rats. PAH clearance was also significantly increased, but in this instance the difference between the two groups was less.

Two further groups each of eight pregnant rats were used. In the first, pregnancy was terminated on the 13th day by removing the entire uterine contents. In the second group, all foetuses were removed on the 13th day but the placentas were left *in situ*. Inulin and PAH clearances were then determined in both groups on the 18th day after mating. Values for inulin clearance were found to be significantly higher in the group in which the placentas had been left, compared with the group in which, in addition, the placentas were removed.

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The protective action of an algal polyanion against experimentally produced peptic ulceration in the guinea-pig.

By W. ANDERSON and J. WATT. *Department of Pharmacology and General Therapeutics, and Department of Pathology, University of Liverpool*

In a suitable acid environment sulphated polysaccharides in low concentrations interfere with proteolytic digestion (Babkin & Komarov, 1932; Levey & Sheinfeld, 1954). A degraded polysaccharide, *Ebimar*, retaining about 30% sulphate and exhibiting low viscosity in solution, has been prepared from the red seaweed, *Chondrus crispus*. It was used to investigate the role of pepsin in experimentally produced peptic ulceration.

TABLE 1

Treatment	No. of animals	No. with ulcers		Severity of ulceration	
		Duodenal	Gastric	Duodenal	Gastric
Control (water)	12	12	11	+ + + +	+ +
Ebimar	12	0	8	0	+

After histamine stimulation of gastric secretion in guinea-pigs (Watt & Wilson, 1958), estimations were made of pepsin and free and total acid in samples of juice removed 20, 80 and 140 min after intra-oesophageal administration of 200 mg Ebimar in 2 ml. water. Compared with control values obtained 14 days previously for the same animals, the mean reduction in peptic activity at the stated intervals was 70, 59 and 37%, respectively. There was only slight reduction in acidity.

These results suggested that the substance might have a protective action against experimentally produced peptic ulceration. Two groups, each of twelve guinea-pigs, received histamine diphosphate (30 mg/kg) in a beeswax/oil base intramuscular, preceded by promethazine 7·5 mg intraperitoneal. One group was given 2 ml. of 20% Ebimar solution, by stomach tube, at 3-hourly intervals from 6 hr before to 24 hr after the injection of histamine. The other group (control) was given 2 ml. water at the same intervals. The animals were then killed and the severity of the ulceration assessed.

Table 1 shows that Ebimar had a protective effect against peptic ulceration, particularly of the duodenum. With toluidine blue, Ebimar was demonstrated in the mucus lining the stomach. We tentatively conclude that complex formation occurs between mucoprotein and Ebimar which may contribute to its protective effect.

(Ebimar was prepared in the Research Laboratories, Evans Medical Supplies, Ltd, Liverpool).

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The body temperature of the hippopotamus. By C. P. LUCK and P. G. WRIGHT, *Department of Physiology, Makerere College, Kampala, Uganda*

During a recent population-control operation in the Queen Elizabeth National Park in Uganda the opportunity arose to measure the body temperature of the hippopotamus (*Hippopotamus amphibius*). In the day-time animals were driven from wallows but at night they were already ashore. They were killed by a brain shot at close range and immediately thermistors were placed in the rectum at a depth of 12 in. (30 cm) from the anus and on the skin of the back and the belly.

In two groups of twelve and thirteen animals, each spread over 24 hr but separated by 6 months, the mean rectal temperature was 95·7° F (35·4° C; S.D. 0·7° F). The rectal temperature of wallowing animals was usually slightly below this average and in animals on land it was usually slightly above the average, but there was no obvious variation with the time of day. The belly-skin temperature in wallowing animals was considerably lower than the rectal temperature, but the unsubmerged back-skin temperature rose during the day until it was similar to or exceeded the rectal temperature. In animals on land at night, or those which had been completely submerged before death, there was little difference between the two skin temperatures. The air temperature at night was about 65° F (18·4° C) rising during the day to 90° F (32·2° C) in the shade. The wallow water temperature was 75–80° F (23·8–26·1° C).

The constancy of the rectal temperature is in contrast with that of the white rhinoceros (*Ceratotherium simum*) (Allbrook, Harthoorn, Luck & Wright, 1958), which is an animal similar in body shape and size, under similar land environmental conditions. The possible relationship between habitat and mode of temperature regulation will be discussed.

We wish to thank the Director and the Trustees of the Uganda National Parks for permission to make these observations.

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Molecular configuration of monosaccharides and insulin action.

By J. M. Peterson. *Physiology Institute, University of Cardiff*

Goldstein, Henry, Huddlestun & Levine (1953) postulate that a definite configuration, similar to that of D-glucose, at the first three carbon atoms of the monosaccharide molecule determines the action of insulin in increasing the distribution space of the sugar in the body.

The earlier work of Herring, Irvine & Macleod (1924) provides evidence that it is the configuration at carbon atoms 3, 4 and 5 that determines the ability of a sugar to remove hypoglycaemic convulsions resulting from insulin. These two views on insulin action are not obviously reconcilable. The molecular configuration on which the postulate of Goldstein and his colleagues is based is found in D-galactose and the observation by Levine, Goldstein, Klein & Huddlestun (1949) that insulin increases the space for this sugar has been confirmed.

D-Mannose, however, has not the required configuration yet it gives the following results when injected intravenously together with an equal quantity of D-glucose in saline solution, into the anaesthetized dog whose abdominal viscera, including liver and kidneys, have been removed. (1) It behaves like D-glucose in (*a*) the rate of its disappearance from the blood and (*b*) the increase in rate of disappearance after injection of insulin; (2) after injection of insulin the concentrations of the two sugars in the blood tend to become the same and when this occurs they remain of equal value as they fall to a level which would be incompatible with life were the animal dependent on the glucose alone.

D-Mannose injected intravenously rapidly removes hypoglycaemic convulsions due to insulin not only in the intact dog but also in the decerebrate cat with abdominal viscera, including liver and kidneys, removed. This liverless preparation remained in good condition when the concentration of glucose in its blood was < 4 mg/100 ml. and that of mannose 146 mg/100 ml. (An observation by Mann & Magath of the ability of mannose to remove insulin convulsions in the liverless dog is referred to by Herring *et al.* (1924).) It is difficult to see, from the evidence at present available, how these epimeric aldohexoses, D-mannose and D-glucose, so similar in behaviour, can be classified differently as regards their responses to insulin.

The mannose used, from Messrs Kerfoot, is inert to the action of glucose oxidase.

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On the distribution of synaptic end-feet (boutons terminaux) in the central nervous system of the cat.

By G. B. David, A. W. Brown and K. B. Mallion. *M.R.C. Neuropsychiatric Research Unit, Whitchurch Hospital, Cardiff*

The distribution of synaptic end-feet (boutons terminaux) in the C.N.S. of the cat was studied with a new staining method. Thin sections of double-embedded blocks of the C.N.S., processed by a technique described elsewhere (David, 1955), were stained for short periods in stabilized silver diamine, without impregnation with metallic salts.

The end-feet seen in the ventral horns of the spinal cord and in the dentate nucleus of the cerebellum are similar to those in the medulla oblongata, described elsewhere (David, 1957). The greater part of the surface of the post-synaptic neurone is covered by end-feet; there are 10–18 of these per $100\mu^2$, and their mean diameter (calculated from measurements of 404 end-feet on high definition photomicrographs) is $1{\cdot}27\,\mu$. These figures are in close agreement with those published by Wyckoff & Young (1956) for carbowax-embedded material. Since the dendrites and axons are found to contain, proportionately, as many end-feet as the perikaryon, it is reasonable to assume that a large neurone of the spinal cord can receive nervous impulses across 2000–3600 synapses On the Purkinje neurones of the cerebellum there are also many end-feet, but these are much smaller. The end-feet in the cerebral cortex are smaller still, and can barely be resolved with the optical microscope.

End-feet in the C.N.S. are not difficult to stain when the following requirements are met: (i) rapid fixation; (ii) preservation of lipoproteins; (iii) avoidance of shrinkage and distortion during histological processing.

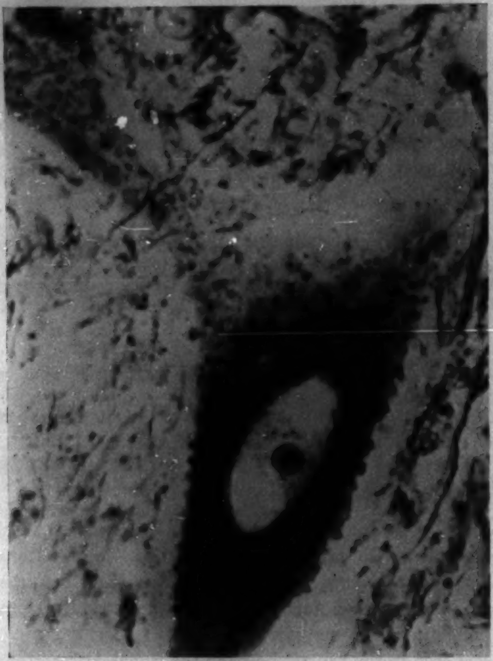

Fig. 1. End-feet on the surface of a neurone of the ventral horn of the cervical enlargement of the spinal cord of the cat. Photographed under homogeneous immersion, N.A. 1·30, at 610 mμ.

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Examination of blood and urine for melanophore expanding substance.

By J. N. KARKUN, R. A. MAIN and G. M. MITCHELL. *Department of Pharmacology, Institute of Preventive Medicine, Cardiff*

An isolated preparation of atria with sympathetic nerves. By S. Huković. *University Laboratory of Pharmacology, Oxford*

The enzymes associated with acetylcholine synthesis in rabbit atria. By A. S. Milton. *University Laboratory of Pharmacology, Oxford*

The effect of Dibenyline on the vascular response to sympathomimetic amines in the forearm. By M. J. Allwood and Jean Ginsburg. *Sherrington School of Physiology, and Department of Medicine, St Thomas's Hospital Medical School, London, S.E.* 1

The effect of the adrenergic blocking agent Dibenyline (phenoxybenzamine) on the response to adrenaline, noradrenaline and isopropylnoradrenaline has been studied in the forearm of healthy young adults.

Blood flow was measured in both forearms under standard conditions by venous occlusion plethysmography (Barcroft & Swan, 1953). Intra-arterial or intravenous doses of the amine under study were given before and after the infusion of Dibenyline (500 μg/min for 6 min) into the brachial artery of one side. Dibenyline in this dosage generally caused a moderate increase in flow on the infused side with no change in the contralateral (control) limb.

When adrenaline (10 μg/min I.V. for 10 min) was given after the Dibenyline the response was modified in the infused forearm but not in the control limb. Blood flow increased initially in both forearms but to a greater extent on the infused side. The flow then subsided in the control forearm though remaining elevated in the treated limb throughout the infusion of adrenaline. The response to intra-arterial adrenaline (2 μg/min for 10 min) was also modified, the transient vasodilatation and subsequent vasoconstriction being replaced by a sustained dilatation. Similar effects have been observed by Whelan (personal communication).

The vasoconstrictor response to noradrenaline in similar dosage was reduced though not abolished after the infusion of Dibenyline. The marked dilator response to isopropylnoradrenaline was little altered by intra-arterial Dibenyline.

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The effect of noradrenaline on vasoconstriction caused by sympathetic stimulation. By J. H. Burn and M. J. Rand. *University Laboratory of Pharmacology, Oxford*

Some cardiological observations on athletes at the British Empire and Commonwealth Games 1958. By F. W. Beswick and R. C. Jordan. *Physiology Institute, Newport Road, Cardiff*

The effect of superior cervical ganglionectomy on the intraocular pressure.

By M. E. LANGHAM and C. B. TAYLOR. *M.R.C. Ophthalmological Research Unit, Institute of Ophthalmology, Judd Street, London, W.C. 1*

In tonometric studies Linner & Prijot (1955) and Lieb, Guerry & Ellis (1958) reported that the intra-ocular pressure of rabbits fell to within 1–2 mm Hg of the episcleral venous pressure 24 hr after excision of the superior cervical ganglion. They attributed this to a decrease of more than 80% in the rate of secretion of the aqueous humour, since they could find no change of the outflow resistance by perfusion or tonographic techniques, and concluded 'that the secretion of aqueous humour involves an adrenergic mechanism'. The aim of these experiments was to verify and extend the above observations by more direct methods for the measurement of pressure and the rate of flow of the aqueous humour.

Six rabbits were subjected to unilateral superior cervical ganglionectomy, and 24 hr later the intra-ocular pressure was measured manometrically under urethane anaesthesia. On the side of the ganglionectomy the mean intra-ocular pressure was $16{\cdot}1 \pm 0{\cdot}65$ mm Hg, and on the control side $22{\cdot}0 \pm 0{\cdot}65$ mm Hg. The mean blood pressure for the series was 114 ± 3 mm Hg. Since episcleral venous pressure in rabbits is 9–10 mm Hg, the mean fall in outflow pressure in the operated eyes was approximately 50%. In three of these animals acetazoleamide, a well-established inhibitor of aqueous humour secretion, was injected intravenously and caused a further fall of at least 3 mm Hg in the intra-ocular pressure of the operated eyes.

The rate of flow of the aqueous humour was measured by a continuous fluorophotometric method similar in principle to that of Goldmann (1950). In this procedure k_{flow} is derived from the course of the plasma and aqueous humour concentrations after a single intravenous injection of fluorescein. In five experiments, 24 hr after unilateral ganglionectomy the values of k_{flow} (min^{-1}) in the operated and control eyes were $0{\cdot}0117 \pm 0{\cdot}0013$ and $0{\cdot}0109 \pm 0{\cdot}0012$, respectively. It therefore seems most improbable that the fall of 50% in the outflow pressure was due to a decreased secretion of the aqueous humour.

The alternative explanation, that ganglionectomy led to a decrease in the outflow resistance, found strong support from further experiments in which the effect of different rates of infusion of fluid on the steady state intra-ocular pressure was determined. In six animals 24 hr after ganglionectomy the slope of the curve of pressure against infusion rates in operated eyes was less than in the control eyes.

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Effect of chlorothiazide on a dibenamine-like compound. By

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The diuretic substance chlorothiazide is generally believed to increase the effectiveness of hypotensive agents, particularly ganglion-blocking drugs (Preziosi, Bianchi, Loscalzo & de Schaepdryver, 1959). An experiment was done to find out whether or not chlorothiazide affected the potency of a haloalkylamine related to dibenamine. Atropinized spinal rats of approximately 250 g weight injected with 5 mg/kg of C_6 were used as test object. The effects of 1 μg/kg of adrenaline and of 0·75 μg/kg of noradrenaline (approximately equivalent amounts) on the carotid blood pressure were recorded twice, and again 15 min after each of a series of increasing doses of diphenoxyethylbromoethylamine HBr (JFA 29), the compound selected for trial. This is an antagonist of adrenaline and noradrenaline of greater potency and shorter action than dibenamine. After the pressor response to adrenaline had been reduced by some 80–90 % no more JFA 29 was injected but the response was tested half-hourly for 5 hr. From the data the ED_{50} (amount effective in producing 50 % response) *vs.* adrenaline and noradrenaline and the ET_{50} (time needed to restore the pressor response to half its initial value) for adrenaline were calculated. Twenty-four rats were used, half having been injected subcutaneously daily for 10 days with 50 mg/kg chlorothiazide. Five rats from each group provided sufficient data to determine duration of action.

The ED_{50} *vs.* adrenaline was increased from 93 ± 40 to 126 ± 87 μg/kg, but so great was the scatter of results that this difference is not statistically significant. For noradrenaline the ED_{50} was increased from 155 ± 67 to 234 ± 165 which is only significant at fiducial limits where $P = 0{\cdot}1$. As far as the duration of antagonism is concerned, the increase of the ET_{50} from 106 ± 56 to 292 ± 89 ($P = 0{\cdot}01$) may be related to the increase in dosage found to be required in the limited groups measurable (190 ± 37 to 450 ± 56). This difference in ED_{90} *vs.* adrenaline is just significant ($0{\cdot}1 > P > 0{\cdot}05$).

Treatment of rats with chlorothiazide, if it has any effect, reduces the potency of a dibenamine-like compound. The increased amount needed to antagonize fully a given dose of adrenaline in rats treated with chlorothiazide exerts a longer-lasting effect than the lesser amount needed to exert a similar degree of antagonism in the absence of chlorothiazide. The haloalkylamines differ in this interaction from the ganglionic blockers.

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Antiadrenaline activity in a series of halogeno-phenylethylamines. By G. W. L. James. *Department of Pharmacology, Welsh National School of Medicine, Cardiff*

N-N'-dimethyl-2-chloro-2-phenylethylamine (Ferguson & Wescoe, 1950) differs structurally from dibenamine in that the molecule is weighted heavily on the β carbon atom of the chain while the substituents attached to the *N* atom are minimal. It has been reported to be a brief, powerful antagonist of adrenaline with additional cholinergic properties. The relation between structure and activity was determined in a series of such compounds. Inhibition of the pressor response to adrenaline 1 μg/kg and noradrenaline 0·5 μg/kg in the atropinized spinal rat injected with 5 mg/kg of hexamethonium was used as test object. The following relations were established. In the parent compound the order of potency, as in 2-halogenoalkylamines like dibenamine, is I > Br > Cl. Replacement of halogen by hydroxyl abolishes activity. Weighting of the alkyl chain reduces activity. The dipropylamine compound is more active than the di-isopropylamine homologue. Fixing the alkyl chain in a piperidine or morpholino group has no great effect on activity. Analogous monoamines show a similar structure-action relation but only 5% of the activity of diamines. Isomers of diamines are equivalent; they produce equal amounts of the same ethyleneiminium ion, the active species (Graham, 1957).

The anti-adrenaline action of these compounds is of immediate onset, short duration and non-competitive nature. The most potent is very active, having an ED_{50} of 10 μg/kg (dibenamine 2 mg/kg) in this test. Various members of the series have powerful muscarinic, nicotinic, local anaesthetic and neuromuscular blocking actions and antagonize histamine.

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The effect of changes in environmental temperature upon body temperature and performance during strenuous exercise. By R. G. Bannister* and J. E. Cotes. *Medical Research Council Pneumoconiosis Research Unit, Llandough Hospital, near Penarth, Glam*

Changes in ambient temperature over the range 15–25° C have little effect upon the body temperature of subjects undertaking moderate exercise (Nielsen, 1938); investigations into the limiting effects of temperature have largely been confined to extreme conditions (e.g. Dill, Edwards, Bauer & Levenson, 1931; Lind, Weiner, Hellon, Jones & Frazer, 1957; Robinson, Turrell & Gerkin, 1945; Smith, 1955). It might be expected that temperature variations even within the range 15–25° C could become a limiting factor when subjects are performing very strenuous exercise, or participating in major athletic events.

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This study is an attempt to assess the role of ambient temperature variations under these conditions. The subjects were two British international athletes (S.E. and M.H.) and one Kenyan athlete (A.O.) who had been in Britain for one month. Measurements were made of body temperature (Mead & Bonmarito, 1949), exercise ventilation and energy expenditure during strenuous treadmill exercise, at two levels of ambient temperature. Pulse rates were not recorded. Before exercise each subject performed his usual warm-up out of doors. In each instance the higher temperature study was done first. The lower temperature study was done on a different day in the first two subjects: in the third both were done on the same day. The findings are given in Table 1.

Table 1. **Ventilation, energy expenditure and rectal temperature of subjects exercising on the treadmill at two levels of room temperature**

				Body temp. (° C)			Room temp. (° C)	
Subject	Exercise duration (min)	Energy expended (kcal/min)	Ventilation (l./min B.T.P.S.)	Resting	Rise due to exercise	Rise per kilocalorie increase in energy expenditure	Wet	Dry
S.E.	40	17·92	69·7	37·1	2·1	0·136	22·2	26·1
	45 +	17·01	67·4	—	1·6	0·110	15·3	16·2
M.H.	40	19·54	84·6	36·7	3·7	0·202	20·5	24·4
	45 +	19·06	76·6	—	3·1	0·173	15·3	17·2
A.O.	40	17·06	62·0	37·1	2·1	0·136	20·5	25·0
	45 +	—	—	—	1·9	0·122	15·3	16·7

All three subjects were relatively comfortable and could have exercised further at the lower temperature: at the higher temperature subjects S.E. and A.O. were at the limit of their endurance after 40 min; subject M.H. was glad to stop after 40 min but only confessed to being bored. In each subject the rise in rectal temperature was greater at the higher temperature (mean rises 2·6 and 2·2° C, respectively); the rise per unit increase in energy expenditure was within the range found for average subjects (Cotes & Meade, 1957). The rectal temperature of subject M.H. rose highest and was 40·4° C (104·7° F).

These findings suggest that variations in ambient temperature within the normal range can affect athletic performance in subjects with a normal body temperature response to exercise.

We suggest that this may be one factor contributing to the disappointing performance in the six-mile event at the 1958 Empire Games, Cardiff, when the maximum dry-bulb shade temperature was 25·6° C.

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A model to illustrate the effects of noise at a sensory nerve terminal.

By A. J. Buller. *Sherrington School of Physiology, St Thomas's Hospital, London, S.E.* 1

Following the suggestion by Katz (1950), the investigations of Buller, Nicholls & Ström (1953) on frog muscle spindles indicated an irregularity in the time intervals between successive impulses of an afferent discharge which, it was postulated, was due to thermal noise at the sensory terminations. However, calculation of the theoretically expected interval irregularity proved to be impossible, and a model was built to demonstrate the disrupting influence of noise on an otherwise regular series of pulses.

The biological results of Buller *et al.* (1953) could be well matched by the model providing certain assumptions were made. First, that in the absence of thermal noise at the sensory terminations, the rate of afferent impulse discharge would be directly related to the degree of terminal depolarization, between the limits of threshold depolarization and maximal depolarization (cf. Katz, 1950). Secondly that, again in the absence of thermal noise, threshold depolarization of the sensory terminal would lead to an afferent discharge at a rate of 15–20 impulses per second. This latter assumption implies that a muscle spindle firing at a mean rate of less than 15 impulses per second does so because some noise transients exceed threshold depolarization and lead to impulse initiation.

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Simultaneous measurement of propagation velocity of action potential and contraction wave in frog striated muscle.

By D. V. Roberts. *Physiological Laboratory, University of Liverpool*

Buchthal & Knappeis (1943), stimulating isolated single fibres of the semitendinosus muscle in *Rana esculenta* and *R. temporaria*, found that the contraction wave travelled at 0·32 m/sec at 20° C. Abbott & Ritchie (1951), however, observed a propagation velocity of 0·51 m/sec at 0° C for the contraction wave and 0·56 m/sec at 0° C for the action potential in the whole sartorius muscle of the English frog. The experiments to be presented were carried out on whole sartorius muscles of the English frog, dissected out and fastened down in a bath of Ringer's solution with enough tension applied to the ends of the muscle to hold it at its original length before removal from the body. Small pins were put through the connective tissue along each border of the muscle for about half its length from its pelvic origin. Then the next quarter of the muscle was allowed to fall into a loop by reducing the tension exerted on the tendon, and finally the last quarter of the muscle was pinned and extended like the first half. A fibre was chosen which ran on the surface of the muscle over its whole length and was visible in the first half and in the last quarter of the muscle. Two glass capillary micro-electrodes were inserted into this fibre at points a known distance apart in the first half of its length, and were connected through a change-over switch to a positive voltage pulse generator, so that a pulse could be applied to each electrode in turn. A third micro-electrode was inserted into the same fibre in the last quarter of its length to record the action potential. Movement of the fibre was recorded by a transducer (RCA 5734) hooked on to connective tissue close to the recording micro-electrode. The fibre was then excited by each stimulating electrode in turn, and the action potential and contraction resulting in each case were recorded on a double-beam cathode ray tube. The photographic records were superimposed and the temporal difference corresponding to the spatial difference between the two points of stimulation was found by measuring the time interval between the two stimulus artifacts. Propagation velocities measured by this method for action potential and contraction wave were 2·52 m/sec at 21° C, 1·06 m/sec at 9° C and 1·39 m/sec at 4° C, a different fibre being used in each case.

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The effects of some metabolic inhibitors on biliary excretion of water, endogenous bile pigment and bromsulphalein. By S. L. Stone. *Physiology Institute, University of Cardiff*

Cooke, Lawler, Calvin & Green (1952) have shown that secretion plays a part in the formation of bile, and it was therefore of interest to study the effects of metabolic inhibitors on the process of bile formation.

Dogs were anaesthetized with pentobarbitone sodium, the cystic duct was doubly ligated and the gall-bladder drained. The common bile duct was cannulated and hepatic bile samples were collected serially. Cats were prepared in the same way, except that anaesthesia was induced with ether and maintained with pentobarbitone sodium. All the inhibitors were injected intravenously. Bromsulphalein (BSP) was administered as a single intravenous injection in a dose of 5 mg/kg.

Dog. 2:4-Dinitrophenol (5 mg/kg) produces a pyrexia and a choleresis. The concentration of bile pigment is reduced but the total amount of pigment excreted is increased. The time of appearance of BSP in the bile, and its maximum concentration, do not differ from those observed in control animals.

Phlorrhizin (100–200 mg/kg) produces a short-lasting choleresis (Jenner & Smyth, 1956). Both concentration and amount of endogenous pigment are reduced. The maximum concentration of BSP is greatly reduced.

p-Chloromercuribenzoic acid (36 mg/kg) produces a prolonged hypotension, a reduction or no change in bile volume, and a slight increase in endogenous pigment concentration. The excretion of BSP does not differ significantly from normal.

Cat. 2:4-Dinitrophenol (4–12 mg/kg) produces little pyrexia and a very slight choleresis at higher dosage. There is an increase in endogenous pigment excretion after a long latent period. The time of appearance of BSP and its maximum concentration do not differ from normal.

Phlorrhizin (250 mg/kg) produces a slight choleresis, but the amount of endogenous pigment and its concentration are greatly reduced. The maximum concentration of BSP is markedly reduced.

Benemid (120 mg/kg) produces an increase in the rate of bile formation, with a reduction in concentration but no decrease in the amount of bile pigment excreted. The maximum concentration of BSP in the bile is below normal. These results are consistent with the findings of Goetzee, Richards & Tindall (1958) and Stone (1958) in the dog.

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The effect of temperature on the buffer value and the titration curve of oxyhaemoglobin solutions.

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